PLANT PHYSIOLOGY

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PLANT PHYSIOLOGY

VOLUME 32 JANUARY, 1957 NUMBER 1

THE RISE OF SAP IN A TROPICAL LIANA[1,2]

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It was discovered in a Palestine orchard that citrus trees do not necessarily wilt when the trunk is cut from opposite sides at different levels so that every vessel is severed (2). Similar observations have been made on several other species of trees (3). Furthermore, it is known that every transpiration column throughout the cross section of a tall grapevine can be broken by air, without retarding the water uptake. When the air breaks are too extensive the transpiration slows down considerably, but it still may be sufficient to prevent wilting or even to permit a wilting plant to recover (4).

It is common to these cases that air breaks existed in all or most of the active vessels throughout a full cross section of the stem, so that the transpiration stream for some distance, at least, must have run only through the interstices between the vessels.

These phenomena have an important bearing on the cohesion theory, as well as on the relation between vascular and interstitial sap flow in the stem, and it has been our endeavor in the present investigation to elucidate the situation by experimental studies of hydrodynamic conditions in a liana.

THEORETICAL CONSIDERATIONS

Prior to the present investigation a simple concept of the hydraulic system in a vine was developed, which proved useful as a working hypothesis. The ideas were based on the following data from the grapevine (4).

In the first place, when a short piece of stem was trimmed from the upper end of a long excised grapevine section, only a small amount of sap ran out at the lower end. If another such piece was trimmed from the top, more sap ran out. Consequently the sap must have been hung up at the upper end, very likely because the pits in the vascular walls would not allow an air-water meniscus to pass. Secondly, in a piece of stem one decimeter long the sap in the vessels could be easily shaken out, but the sap in the interstices (tracheids and walls) could not be dislodged by centrifugation even at $1700 \times g$. This sap was freely motile, however, because it could be squeezed out at the ends by pressing the wood. We may interpret this to mean that the sap is freely movable within the fixed volume of the rigid interstitial framework, but cannot be dislodged from it because micromenisci in pit membranes and micropores effectively prevent air from entering the finer structures. In the course of these investigations it was also established that the main part of the transpiration stream runs in the vessels, that there is no low pressure gas phase in or near the conducting vessels, and that there are no metabolic pumps in the stem responsible for the sap ascent.

The data taken by themselves suggest an hypothesis which runs along classical lines, i.e., that the water-conducting xylem in the grapevine stem is essentially a flooded, continuous, micropore system, scattered with elongate macrocavities (vessels). The stem may accordingly be compared to a pipe filled with a sinter of fine sand, throughout which large longitudinal cavities are dispersed (fig 1). If water fills such a system and flows through it, the cavities will offer the paths of least resistance, and through them most of the water will flow. If the hydrostatic pressure is below atmospheric and outside air enters a cavity, this will press the water out of the cavity, but no farther, as the air-water menisci will hang up in the micropores of the cavity walls. The stream of water must now flow past the air-blocked cavity, and the resistance to flow increases. Full flow can then be maintained only at the cost of an increased pressure drop across the resistance (fig 1 c).

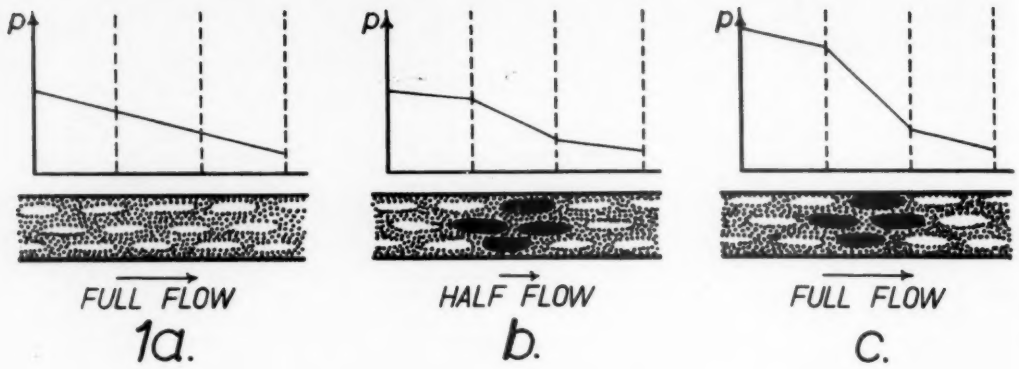
It is the purpose of this paper to report our experimental tests of the above hypothesis.

MATERIALS AND METHODS

MATERIAL: Lianas of many different genera grow on Barro Colorado Island in Gatun Lake, Canal Zone. Sterile vines *Tetracera* sp. belonging to the Dilleniaceae were especially well suited to experimentation because they have large vessels and, unlike the grapevine, produce little, if any, gelatinous exudation from the central xylem. In Central America at least one species of Tetracera is referred to as "bejuco de agua" or "water vine" (5) because quantities of potable sap run out of a stem section when this is rapidly severed from the plant by two slashes with a machete.

[1] Received May 18, 1956.

[2] Supported by grants from the Norwegian Research Council and the Rockefeller Foundation.



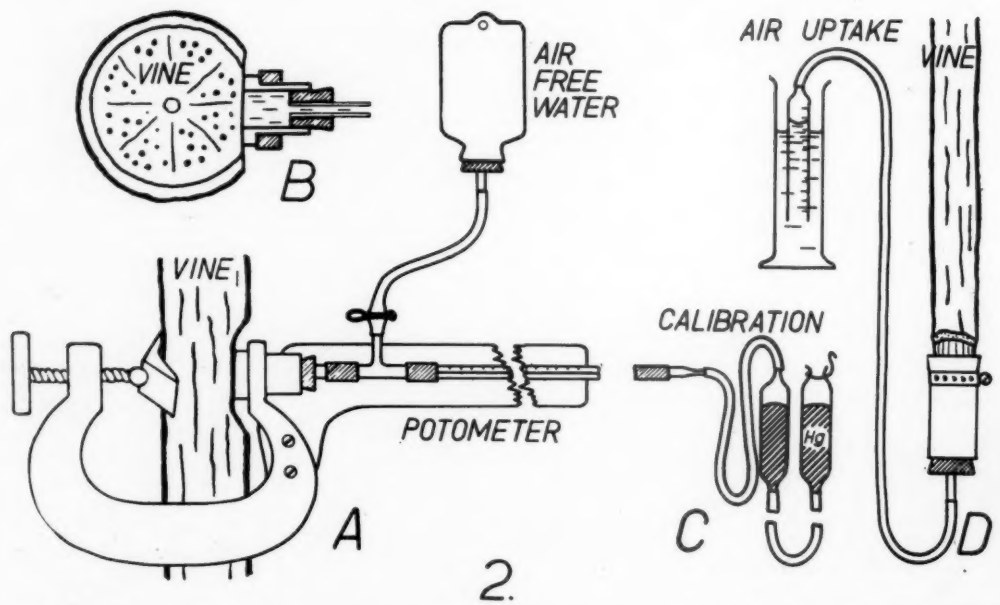


FIG. 1. Flow model of a microporous matrix with longitudinal cavities. Water flow in direction of arrow. a. All cavities water-filled. b, c. Some cavities blocked by air. The curves above show the pressure changes along the tubes.

FIG. 2. A. Potometer assembly mounted on a C-clamp. B. Plastic contact cup on exposed xylem. C. Mercury levelling bulb for pressure calibration of potometer. D. Measurements of air uptake by cut stem. When water uptake is measured the rubber tubing from D draws water directly from the measuring cylinder (cp fig 5).

SOME XYLEM DIMENSIONS: A cross section of a small (1.5 cm thick) Tetracera stem is reproduced in figure 3. Most vessels are between 0.2 and 0.4 mm in diameter. They end blindly after reaching an average of some 1.5 m in length, determined according to the procedure used for the grapevine (4). The vessels are held in a meshwork of tracheids some 10 to 40 microns wide and ranging from less than a millimeter to a few millimeters in length. The walls of vessels and tracheids are richly studded with pits.

METHODS: To test the proposed hypothesis the following measurements were needed: 1) Water and air uptake by the cut stem. 2) Hydrostatic pressure both in vessels and microporous structures.

The total water uptake of a vine cut under water was determined by clamping a hose to the submerged

lower end and connecting it with a measuring cylinder (fig 5). Air uptake (fig 2) was measured by emptying the hose at D and connecting it with an inverted calibrated glass cylinder, which fitted into the water-filled cylinder.

Positive pressures in vessels down to 0.2 atmosphere were estimated with the manometer punch (4).

A record of pressure changes in the microporous structures was obtained by means of a potometer arrangement (fig 2 A, B, C). To apply this, the xylem was exposed and the surface cut flat with a sharp knife. A paste of tooth powder and water was rubbed onto this surface, filling the exposed and opened vessels and providing a microporous contact with the vessel walls which helped to immobilize the air trapped in the vessels. The surface was then covered with vaseline to make it air tight, except for a circle 0.5 cm in diameter over which the plastic contact cup (B) of the potometer was clamped. By loosening the cup stopper the potometer system could be filled with boiled air-free water from a rubber bag without trapping any air bubbles.

The rate of water absorption was measured on the potometer capillary. Assuming the water uptake through the exposed xylem to be a simple process of filtration, i.e, where rate of filtration is proportional to the pressure difference, it should be possible to calibrate the potometer rates in terms of absolute pressure by adding to, or subtracting from, the atmospheric pressure a known pressure, ΔP. The increase or decrease in filtration rate, ΔF, would give the absolute filtration pressure (P) according to the equation $P = \Delta P/\Delta F \times F_1$, where F_1 is the filtration rate at ambient pressure. For each experiment $\Delta P/\Delta F$ would be constant.

In a series of experiments various pressures (ΔP) were applied to the same vine, using a simple mercury calibrator (fig 2 C). The correlation $\Delta P/\Delta F$ was found to be quite linear for each experiment (fig 4). However, when absolute pressures derived by this calibration were compared to those obtained with the manometer punch, the results of the two methods were found to deviate increasingly from one another as the pressure fell below 1 atmosphere. At a pressure in the vessels of about 0.2 atmosphere, recorded directly by the manometer punch, the potometer calibration usually indicated a much higher pressure, i.e, around 0.6 atmosphere.

A similar discrepancy was found independently by another test. In this case the lower end of a cut-out vine section 6 m long was held at different heights, while potometer measurements were made at the upper end. Again the pressure changes indicated by the potometer fell short of the true hydrostatic difference.

These discrepancies cannot be disregarded. They mean either that the postulated free communication between the vessels and microporous structures does not exist, or more likely that the potometer method gives results encumbered by a proportional error.

Results

Effect of Air in the Transpiration Stream: In earlier experiments on tall grapevines, when a cut

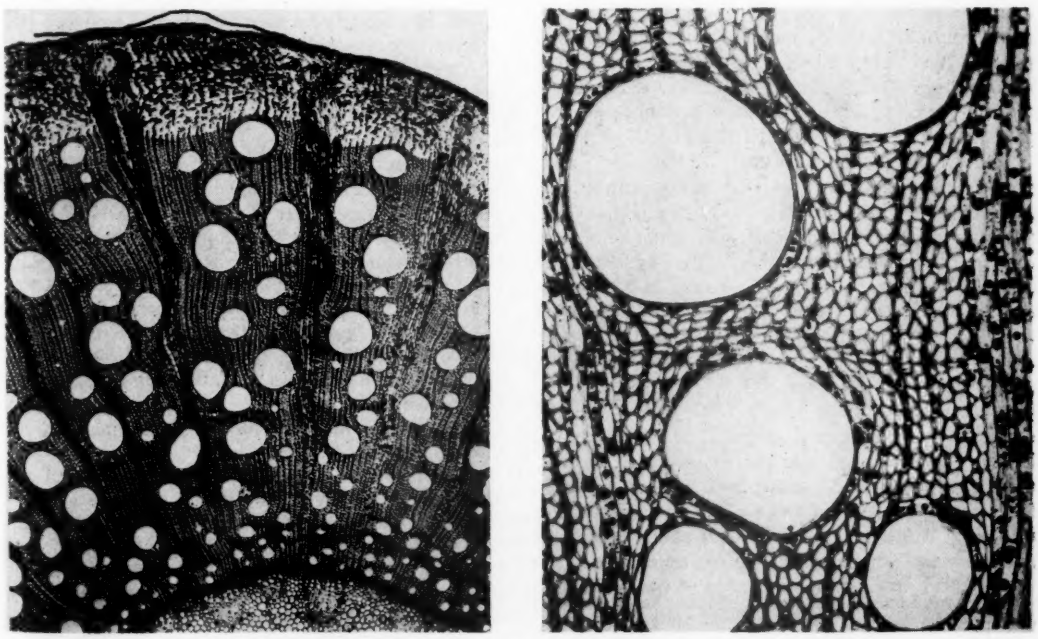


Fig. 3. Cross section of stem of Tetracera liana. Magnifications 20 times and 100 times.

stem was given a sequence of water-air-water the rate of uptake was in all three steps the same, i.e, the water uptake remained unchanged in spite of gross air embolism in the vessels (4). The experiment was repeated on Tetracera lianas with the same results. In this case the events were subjected to closer study by measuring 1) the rate of water intake, 2) the rate and amount of air taken in, 3) the rate at which water absorption was resumed, and 4) simultaneously the pressure in the microporous structures as indicated by a potometer. The result of a typical experiment is plotted in figure 5.

At B_1 the vine was cut under water, resulting in a reduction of the potometer rate from A_1B_1 to B_1C_1, as the vine now received water at a pressure of 1 atmosphere. The cut vine was provided with a tube and the water uptake was registered. At CC_1 the stump received air, and it will be seen that the air, following the receding water columns, disappeared into the vine at the same rate as the water (BCD). After a few minutes, however, the intake of air suddenly stopped (D), and at the same time the potometer rate increased four times (D_1E_1), indicating a pressure drop of similar magnitude. This can only mean that the menisci of the receding water columns in the vessels were stopped by the microporous pit membranes in the walls of the blindly terminating vessels. The continued evaporation from the leaves could now exert its full force on the blocked water, producing the pressure drop at D_1.

At EE_1 water uptake was permitted to resume and it proceeded at a near pre-air rate (EF), but with a potometer rate (E_1F_1) now three times higher than before the admission of air (B_1C_1). Since the water consumption was essentially undiminished, in spite of air-blocked vessels, the rate of flow in the micropores must have increased, causing the observed drop in pressure, i.e, exactly in accordance with the predictions based on the model (fig 1 c).[3]

Effect of Simple Cutting: We may now interpret the potometer readings when a transpiring vine is cut (fig 6 I). As the stem is severed (B) the low pressure (AB) near the cut in the upper section of the vine rises to near atmospheric (BC) while the sap columns in the vessels recede drawing in air behind them. When the air-water menisci reach the pits in the vessel end walls they cannot pass, and the water movement is now completely blocked throughout the cross section of the stem (C). From then on the pressure in the sap falls rapidly (CD), due to the pull exerted by the continued evaporation loss from the leaves. When applied near the cut end the manometer punch registered atmospheric pressure simply from hitting the severed and air-filled vessels. Higher up, i.e, at a distance above the cut of more than an average vessel length, the punch registered nearly the

[3] The total amount of air drawn into the stem corresponds in volume to the maximum amount of water which is discharged from the lower end of a long vine section when a piece as long as the average vessel length (ca 1.5 m) is trimmed from the upper end (4).

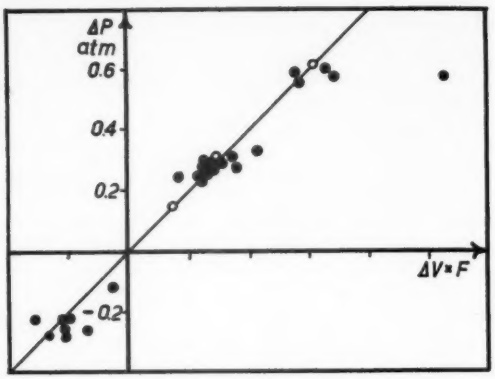


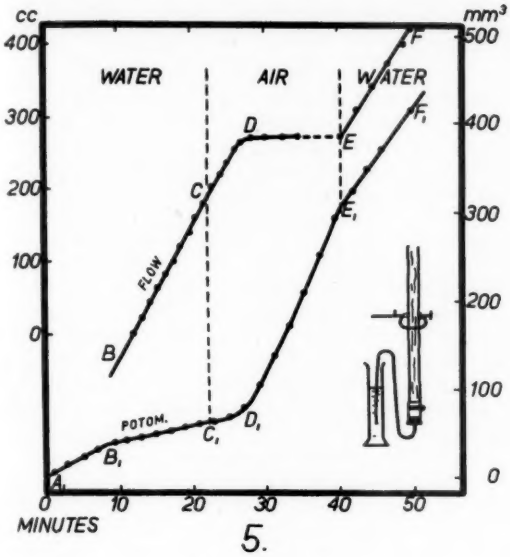
Fig. 4. Relation between calibration pressure (ΔP) and the corresponding change in potometer filtration rate (ΔF). Open circles. Filtration rate obtained by hydrostatic weight of water. Abscissa in arbitrary units which varied for each set of determinations.

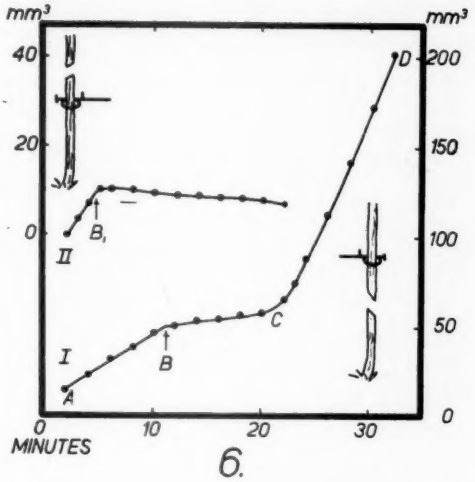
water vapor pressure from hitting only sap-filled, unsevered vessels.

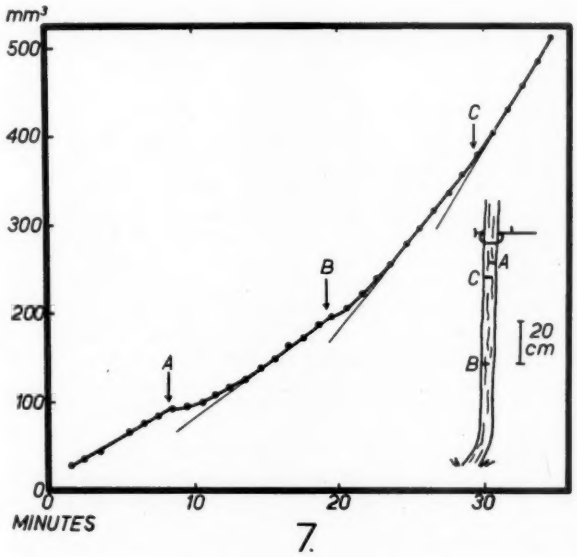
In the lower stump we find initially a pressure rise towards atmospheric as the water recedes into the vessels due to the elastic rebound of the stem and root system. When the menisci are stopped by end walls, the pressure typically drops, only to rise again through refilling from the roots until the stump starts bleeding. In one vine used for such an experiment (fig 6 II) the pressure was high due to prolonged rains, and bleeding from the lower stump started almost immediately so that sap flowed into the potometer and reversed the curve.

Effect of Double Cut: In one series of experiments cuts were made at different levels with a saw on opposite sides of the stem (fig 7). Such treatment does not usually produce wilting (cp above). When the first cut was made half way through the vine (A) there was a marked drop in pressure above the cut, indicated by the higher potometer rate. With the next cut (B) the pressure dropped further, as most vessels between A and B had now been opened to air and between the cuts the transpiration stream was forced to run mainly through the microporous structures. When the distance between the cuts was decreased by the third cut (C) the area through which the water could travel in the micropores was greatly diminished, and the pressure dropped even further. The minimum was reached when the vine was cut through completely.

In a vine with a double cut only those vessel elements which are severed by the saw become inoperative. Below and above this the vessels are full of sap with conduction intact, figure 8 (cp (1), figs 17 and 18). The fact that wilting does not occur means simply that the transpiration stream has force enough to overcome the greatly increased resistance by developing an increased pressure gradient across the region of inactivated vessels, such as recorded by the potometer.







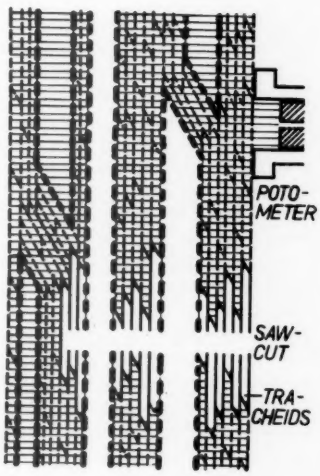


FIG. 5. Measurement of water-air-water uptake by cut vine and simultaneous measurement of potometer filtration rate. The rate of water uptake of the vine is nearly the same before (BC) and after (EF) the air plug (CE), but the potometer rate (B_1C_1) increases fourfold (E_1F_1), indicating a similar pressure drop.

FIG. 6. Changes in potometer filtration rates produced by cutting the stem in air (at B and B_1). I. Potometer on upper section of cut vine. II. Potometer on lower section of cut vine.

FIG. 7. Increase in potometer filtration rate produced by cutting the stem half way through at different levels (A, B, C), indicating a similar drop in pressure. Location of cuts drawn to scale in lower right-hand corner.

FIG. 8. Diagrammatic representation of the effect of a saw-cut in a stem with sap pressure below one atmosphere. Water-filled structures shaded horizontally. Large channels are vessels.

Discussion

We have demonstrated in the present investigation that air introduced into the transpiration stream of a liana stays confined to those elements which are severed (vessels, tracheids), and does not spread into other compartments through pits or micropores in the walls. This verifies experimentally a basic assumption made by Dixon (1).

When gas is admitted to vessel compartments containing sap at subatmospheric pressure the sap is sucked out of them and they are eliminated as water conductors. This produces a local increase of resistance, as the transpiration stream is now denied channels of easy flow and must pass around them in the microstructures. Normal flow can then only be maintained at the cost of an increased pressure drop across these breaks. Such a drop has been demonstrated in every case of experimentally produced gas breaks.

Potometer experiments on transpiring vines have shown that the pressure gradient to the outside often increases three to four times when the vine is cut or when air is given to an already cut vine. In many cases we know from direct measurements with the manometer punch that the vessel pressure before the cut was, say, 0.2 or 0.8 atmosphere and hence, assuming the same pressure in the vessels as in the microstructures, several atmospheres' tension must have developed in the stem above the break. As this was measured at ground level one might be tempted to extrapolate it to reach some – 10 atmospheres at the top of a liana 40 meters tall. It is true that accurate, positive hydrostatic gradients have been measured in grapevines in the spring and at night, but we failed then, and have failed also in our present Tetracera vines to show that such a gradient exists during transpiration. In either case, however, one may suspect that such will be found when a proper technique is applied. It should also be emphasized that there is no direct demonstration so far of equality of pressure in vessels and microstructures, no matter how likely this may also seem to be. One may temper rash conclusions by pointing out the fact that experimental evidence for the stability of the presumed cohesive columns in the vessels is rather negative and that no mechanism is known which could repair breaks high in the vine (4). Even in the wet season at Barro Colorado, when measurements were taken at night in pouring rain, the manometer punch failed to give high enough pressures at ground level to account for the repair of vascular gas breaks 20 meters up and higher.

One seemingly basic obstacle to the concept of the cohesive lift of water in the vessels has now been overcome, however, by demonstrating that in vines air breaks stay confined, and hence they will not seriously damage a cohesive system.

Summary

Pressure-flow relations have been studied in the stem of a tropical liana (*Tetracera* sp.). It was shown by potometer measurements that whenever an air break is introduced into the vessels of a transpiring vine, e.g, by a double cut, or by cutting it off completely, the pressure above the cut always drops drastically.

When a cut liana takes up water from a burette and then is given air the air is drawn in at the same rate as the water, but soon stops abruptly, i.e, the receding water columns hang up. From this point on we observe the above-mentioned pressure drop. The air which enters the severed vessels is evidently stopped by the pit membranes in the vessel walls which do not allow an air-water interface to pass. As the vessels are of limited length, the air does not spread. The vessels represent the paths of least resistance to the transpiration stream, and whenever vessels are severed a subatmospheric hydrostatic pressure will empty them, thus rendering them inoperative for water conduction. This means a locally increased flow resistance which results in the recorded pressure drop. There are good indications that the drop may reach cohesive values.

The results demonstrate that air breaks in the vessels and tracheids produced by various cuts stay confined in the severed units and that the transpiration stream simply moves around these obstacles in the intact units, as postulated by Dixon. Hence the idea of cohesive lift is not incompatible with air breaks in the transpiration stream.

We are much indebted to A/S Thor Dahl, Knut Knutsen O. A. S. and the State Institute for Whale Research in Norway, which provided the transportation for personnel and equipment to Panama and back. Our thanks are due to Professor Dr. Johan T. Ruud of the University of Oslo for his aid in arranging the expedition and to Cand. Real. Paul Berg for preparing histological sections. The instrumentation was ably made by Mr. Odvar Iversen of the Institute of Zoophysiology, University of Oslo. Our cordial gratitude goes to Dr. James Zetek, Director of the Canal Zone Biological Area, for unfailing help and inspiration and to Mr. Francisco Vitola for his assistance on Barro Colorado Island.

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STUDIES ON THE PHOTOSYNTHETIC REACTION. III. THE EFFECTS OF VARIOUS INHIBITORS UPON GROWTH AND CARBONATE-FIXATION IN CHLORELLA PYRENOIDOSA [1,2]

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The chromatographic-radioautographic technique for the analysis of soluble, intracellular metabolites has been eminently successful in its original application—that of determining the mechanism of carbon dioxide fixation in green plants (1). To the extent that the absence of light can be considered an inhibitor, the technique is thus shown to be valid for the study of inhibition mechanisms.

The first application of the technique to inhibition by a chemical agent (2) appears in the negative, though apparently valid, result of adding malonate to photosynthesizing Scenedesmus. Various extensions of this technique have appeared (3, 6, 10, 14). The work of Havinga et al (6) on the effects of antibiotics upon Scenedesmus was the first systematic attempt to apply the technique to chemical inhibitions. Except for some small but essential modifications of procedure, this work is the model for the present paper.

The present study is negative with regard to its ultimate purpose of finding specific inhibitors of photosynthesis, but there remains a body of data on the effects of various inhibitors upon growth and carbon dioxide fixation in Chlorella.

MATERIALS AND METHODS

ALGAE AND MEDIA: *Chlorella pyrenoidosa* Chick (American Type Culture Collection No. 7516) were grown in pure culture, either on agar slants (4) or in continuous culture shake flasks, using the major nutrients of Myers (7) (except for supplying the iron as the ethylenediamine-tetraacetic acid complex), the modified micronutrients of Arnon (8), and 5 % CO_2 in air.

For growth tests, algae were scraped from agar slants and diluted with sterile water to a concentration of 3 to 5×10^7 cells/ml. For measurements of radiobicarbonate fixation and for the preparation of extracts for chromatography, the shake flask harvests were centrifuged (30 min, 800 ref at tube tip) and resuspended to 2 % concentration by volume in the experimental medium.

This experimental medium contained: 5×10^{-3} M KNO_3, 2×10^{-3} M $MgSO_4$, 0.25×10^{-3} M $Ca(NO_3)_2$, 2.3×10^{-6} M $Na_2B_4O_7$, 1.9×10^{-6} M $MnCl_2$, 1.0×10^{-6} M Na_2MoO_4, 0.17×10^{-6} M $CoCl_2$, 0.16×10^{-6} M $CuCl_2$, 0.15×10^{-6} M $ZnCl_2$, 1.6×10^{-8} M $Fe(NH_4)_2(SO_4)_2$, and 0.02 M potassium phosphate buffer. The iron at 1000-fold concentration was autoclaved separately in acidic solution. The phosphate buffers used were (a) K_2HPO_4 and KH_2PO_4 in the molar ratio 1:19, (b) the same salts in the ratio 3:17, and (c) K_3PO_4. In a complete growth mixture, after equilibration with 5 % CO_2, the pH's were 5.1, 6.0 and 7.4. The first of these solutions is rather poorly buffered and variations of as much as 0.4 pH unit were sometimes observed.

GROWTH TESTS: A 24-tube aeration apparatus was built according to the general plan of Myers (9). The tubes were autoclaved with 5.0 ml of double strength experimental medium plus 3.5 ml of water. While still warm, the tubes containing alkaline medium were aerated with pure CO_2 to redissolve precipitated material. All tubes were equilibrated with 5 % CO_2 in air before adding the 0.1 ml of iron solution, 1 ml of pH-adjusted, appropriately sterilized inhibitor solution (or water) and 0.5 ml of the aqueous algae suspension. To prevent evaporation loss during the 48-hr growth test, the aerating gas was first passed through water. After temperature equilibration with the light sources the suspensions inside the tubes had a temperature of 25° C. Optical densities were measured twice a day at 610 $m\mu$ by means of a Lumetron colorimeter. For non-inhibited control runs the optical densities were initially in the range 0.10 to 0.15, and after 48 hours they were in the range 0.55 to 1.30. Separate non-inhibited controls were used at each pH. In the first run with any inhibitor a control without algae was included to note possible interference with colorimetric readings by either the inihibitor itself or its possible decomposition products. Tubes were always run in duplicate, and their optical readings stated in terms of percentages rarely differed by more than three or four units in either initial or final readings.

The inhibition data of table I are stated as percentages (compared to the non-inhibited controls) based on optical density increments from the start of the experiment to 40 hours. The 40-hr period is arbitrary for the sake of the table. Actually, day to day comparisons of results are improved by using either the 40 or the 48-hr period, according to which happens to give control run optical densities nearest an arbitrary density. This is true because the day to day differences in non-inhibited growth curves are primarily in the lag period.

CARBONATE-FIXATION TESTS: The constant temperature bath (25° C) was a flat vase connected to a constant temperature reservoir by a circulating pump. Light from a 300 w reflector spot lamp had a 7-inch path including 5 inches through water. The reaction vessels were flat centrifuge tubes (Corning No. 8520) calibrated at the 5.0 ml volume.

All tests were carried out in the pH 6.0 experimental medium. To insure an aerating gas of uniform composition for any one series of experiments, the 5 %

[1] Received June 19, 1956.
[2] This work was supported by the Charles F. Kettering Foundation.

CO_2 in air was necessarily supplied from a single cylinder of compressed mixture. The resuspended algae were stored in a test tube under experimental conditions, aliquots being withdrawn as needed. In-

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE GROWTH OF CHLORELLA PYRENOIDOSA

INHIBITOR	CONC	% INHIBITION		
		pH 5.1	pH 6.0	pH 7.4
Antibiotics				
Azaserine (o-diazoacetyl-L-serine) (Parke, Davis)	0.001 M	58	19	0
	0.002 M	81	79	28
	0.01 M	90	83	74
Bacitracin * (61 U/mg)	500 μgm/ml	11	...	0
	1000 μgm/ml	53	...	0
	5000 μgm/ml	100	...	19
Carbomycin (Magnamycin, Pfizer) (1075 μg/mg)	100 μgm/ml	92	98	100
(-)-4-Oxo-2-thiazolidine-hexanoic acid (actithiazic acid, Pfizer) (crystalline)	10 mg/ml	0	0	0
Erythromycin (Ilotycin, Lilly)	36 μgm/ml	0	0	2
	145 μgm/ml	...	15	37
	725 μgm/ml **	63	78	87
Gliotoxin	2 μgm/ml	93	92	95
	2 μgm/ml †	75 †	82 †	59 †
Neothiolutin (Pfizer)	2.5 μgm/ml	21	48	100
	5 μgm/ml	37	75	100
	10 μgm/ml	100	100	100
Netropsin	5 μgm/ml	67	71	70
	10 μgm/ml	100	100	100
Oxytetracycline (Terramycin, Pfizer) (900 μgm/mg)	1 μgm/ml	S ††	S	S
	10 μgm/ml	S	S	S
	50 μgm/ml ‡	...	...	...
Pleocidin	1 μgm/ml	75	75	30
	5 μgm/ml	100	100	100
Polymyxin B (6850 U/mg)	0.5 μgm/ml	30	0	0
	5 μgm/ml	100	100	93
Puromycin (Stylomycin, Lederle)	10^{-4} M	−27 ‡‡	−9	−21
	10^{-3} M	− 5	30	30
	10^{-2} M	100	100	100
Rimocidin Sulfate (Pfizer) (1000 μgm/mg)	100 μgm/ml [a]	11	13	31
Streptomycin	10^{-4} M	77	85	88
	10^{-3} M	87	100	100
Tetracycline (Achromycin, Lederle)	Unstable under conditions of the test.			
Thiolutin (Acetopyrrothine, Pfizer)	2 μgm/ml	82	77	95
	5 μgm/ml	100	100	100

TABLE I (*Continued*)

INHIBITOR	CONC	% INHIBITION		
		pH 5.1	pH 6.0	pH 7.4
Uracil analogs				
5-Bromouracil	10^{-5} M	− 1	− 4	−14
	10^{-3} M	35	31	65
	10^{-2} M	97	99	95
5-Nitrouracil	10^{-3} M	−15	−17	7
Orotic Acid	10^{-3} M	− 3	− 6	− 8
2-Thiouracil	10^{-3} M	31	19	35
Thymine	10^{-3} M	6	−14	8
Plant growth regulators				
2,4-Dichlorophenoxyacetic acid	1 mg/ml	0	0	0
Indole-3-acetic acid	10 μgm/ml	42	30	32
	100 μgm/ml	71	77	68
Sulfonic acids				
2,3-Dihydroxypropane-1-sulfonic acid	10^{-2} M	0	0	0
Isethionic acid	10^{-2} M	0	0	0
Taurine	10^{-2} M	0	0	0
Taurineamide	10^{-2} M	24	15	...

* Due to frothing, the aeration rate in this case was very low.

** Added as a non-sterile suspension.

† Gliotoxin solution aerated for 24 hours before start of experiment.

†† S = Stimulation.

‡ Decomposition products prevent turbidity readings.

‡‡ Negative percentages indicate degree of stimulation.

[a] Complete inhibition for about 24 hours, followed by rapid growth.

hibitor solutions were made up with the experimental medium and readjusted to pH 6.0. By means of long hypodermic syringes this inhibitor solution and plain experimental medium were added to each tube to a total of 0.50 ml. Experiments were run consecutively along with interspersed controls. Using a sharp-tipped pipette each tube received 0.50 ml of the algal suspension. After 5 minutes preexperimental time, the aeration jet was removed and 0.10 ml (0.5 μc) of sodium bicarbonate-C^{14} was injected by syringe. The tube was shaken without removing from the water-bath or from the light beam. After exactly two minutes the experiment was ended by placing the tube in a beaker of boiling alcohol and quickly adding into the tube 4 ml of boiling alcohol. After 5 minutes in the boiling alcohol each tube was adjusted to 5.0 ml and centrifuged. The supernatants were used for triplicate 1-ml planchets for determination of C^{14}-activity. Where necessary, corrections were applied for the self-absorption effect of inhibitors upon the observed radioactivity. In table II the radioactivities for in-

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON THE FIXATION OF CARBONATE BY CHLORELLA PYRENOIDOSA

INHIBITOR	CONC	RADIOACTIVITY OF SOLUBLE EXTRACTS COMPARED TO CONTROLS *
Azaserine	0.05 M	0.95
Bacitracin	1 mg/ml	1.3
	2 mg/ml	2.0
	6 mg/ml	2.7
	38 mg/ml	3.6
Erythromycin	100 μgm/ml	1.18
	500 μgm/ml	1.35
	1000 μgm/ml	1.46
Gliotoxin	3 to 150 μgm/ml **	0.64 to 0.75 **
Netropsin	25 μgm/ml	1.00
	250 μgm/ml	0.96
Pleocidin	5 μgm/ml	0.98
Polymyxin B	100 μgm/ml	1.10
	300 μgm/ml	0.39
	500 μgm/ml	0.00
Streptomycin	10^{-3} M	0.8
	10^{-2} M	0.8
Thiolutin	4 μgm/ml	0.95
	10 μgm/ml	0.80
5-Bromouracil	10^{-3} M	0.8
	10^{-2} M	1.0
2-Thiouracil	10^{-3} M	0.9
	10^{-2} M	1.0

* Controls taken as 1.00.

** The 150 μgm/ml is a suspension. The literature value of solubility is 70 μgm/ml. Within the concentration range tested, inhibitor concentration is not a limiting factor.

hibited experiments are stated as ratios compared to the non-inhibited controls.

CHROMATOGRAPHIC TESTS: The procedure was the same as that for the carbonate-fixation tests, except that the quantities used were ten times as large, and that the reaction medium itself was excluded from the alcoholic extract. This exclusion was accomplished by passing the reaction mixture through a Celite filter bed on a Büchner funnel connected to an adapter with two receivers (13). Within two or three seconds, the filtrate could be collected, the other receiver rotated into position, and algae cells on the filter killed by the addition of boiling 80 % alcohol.

Two-dimensional chromatography of concentrated alcoholic extracts was carried out on Whatman No. 1 paper, oxalate-washed (6) and unwashed, first in phenol-water (72:28 w/w), then in butanol-propionic acid-water (equal volumes of 95 % v/v aq *n*-butanol and 44 % v/v aq propionic acid) (1). Figure 3 of reference 1 is an example of the chromatographic pattern.

Radioactivities were calculated in terms of the percentages of a chromatogram's total radioactivity to be found in each of its spots.

RESULTS

The growth inhibition tests (table I) served to eliminate from further consideration the unstable compounds (rimocidin, tetracycline, and oxytetracycline) and the ineffective compounds (thiazolidonehexanoic acid, nitrouracil, orotic acid, thymine, dichlorophenoxyacetic acid, and all the sulfonic compounds).

By comparison of the effective inhibitor concentrations in the growth test and in the carbonate-fixation test (table II), the growth-inhibiting concentrations of azaserine, bacitracin, erythromycin, netropsin, pleocidin, 5-bromouracil, and 2-thiouracil were found to be ineffective in the carbonate-fixation test. At growth-inhibiting concentrations gliotoxin was a partial inhibitor of carbonate-fixation, but the inhibition of carbonate-fixation could not be increased by increasing inhibitor concentration. Polymyxin B, streptomycin, and thiolutin were fair to good inhibitors of carbonate-fixation, but only at concentrations well in excess of those required for comparable growth-inhibition.

The latter three inhibitors were tested by the chromatographic technique. Considering the distribution of radioactivity upon chromatograms the addition of streptomycin (0.01 M) caused an increase in the radioactivity of sucrose (from 6 to 25 %), and decreases in radioactivity of the phosphate ester area (73 to 55 %), of aspartic acid (7 to 2 %), and of glutamic acid (2 to less than 1 %). Chromatographic patterns in single runs with polymyxin B (400 μgm/ml) and thiolutin (10 μgm/ml) showed no dependable differences from the controls.

DISCUSSION

For various green algae the maximum non-inhibitory dose of bacitracin has been reported as about 1 mg/ml (12). The growth tests of the present studies were essentially in agreement with this, but the short-term measurements of carbonate-fixation at growth-inhibitory levels revealed an unexpected stimulation. The persistence of this stimulation, even at massive dosages of bacitracin (38 mg/ml) was especially remarkable. The explanation of this phenomenon is not apparent.

Erythromycin similarly stimulated short-term carbonate-fixation at growth-inhibitory concentrations, though the effect was not as strong as that of bacitracin. Solubility limitations prevented testing at higher concentrations.

Although the decomposition products of oxytetracycline prevented turbidity measurement at higher antibiotic concentrations, the use of appropriate blanks permitted the qualitative conclusion that oxytetracycline stimulated growth at concentrations of 1 to 10 μgm/ml. Previous reports on the effect of oxytetracycline in growth tests with green algae (5, 12) indicate no inhibition in this concentration range, but they do not report any stimulation. Havinga et al (6) found oxytetracycline and chlortetracycline stimulated the short-term fixation of radiocarbonate at antibiotic concentrations from about 15 to 750 μgm/ml.

The Chlorella used in this study were clearly more

sensitive to gliotoxin than were the Chlamydomonas and Scenedesmus used by Foter et al (5). The unusual aspect of gliotoxin action is its inhibition of carbonate-fixation to the extent of about 30 %, regardless of any gliotoxin concentration in the range 3 to 150 μgm/ml. Since there are many independent mechanisms for the incorporation of radiocarbonate into cellular metabolites, the gliotoxin was probably inhibiting one carbonate-fixation mechanism completely, rather than several mechanisms partially. The data of tables I and II are not sufficient to establish a correlation between growth-inhibition and inhibition of carbonate-fixation, but they do permit the possibility that the primary effect of gliotoxin may be in this inhibited carbonate-fixation mechanism. Even if this were true, it must be pointed out that the term "carbonate-fixation" is meant in its broadest sense: the site of metabolic blockade still could be far removed from any actual carboxylation reaction.

Azaserine, netropsin, and pleocidin had little if any effect on carbonate-assimilation, even at concentrations well is excess of those required for powerful growth inhibition. This was taken as evidence that the inhibition mechanisms were totally unrelated to carbonate-fixation.

Thiolutin and polymyxin B were partially effective as inhibitors of carbonate-fixation; but their clearly greater effect in inhibiting growth was taken as evidence that carbonate-fixation was involved either as a secondary mechanism or as a secondary effect of the primary mechanism. The latter possibility is favored by the failure of the chromatographic tests to reveal any derangement in carbonate metabolism. This is particularly true in the case of polymyxin B, where the quantitative depression of the carbonate metabolism was more obviously at variance with its qualitative integrity.

At streptomycin levels more than sufficient to inhibit growth, the chromatographic study of soluble Chlorella extracts did reveal some derangement in carbonate metabolism. Essentially, streptomycin appeared to have reduced the pool sizes of some major phosphorylated intermediates in photosynthesis, resulting in a more rapid sweep of radioactive carbon into the sucrose pool. A very similar increase of sucrose radioactivity at the expense of radioactivity in the organic phosphate area has been observed in Scenedesmus by Ouellet and Benson (11) as a result of low pH. Unless excess acidity and inhibition by streptomycin are related in some totally unexpected manner, the observed pattern of derangement in carbonate metabolism must be of a rather general and necessarily secondary nature.

Summary

A variety of antibiotics and other possible inhibitors were tested against *Chlorella pyrenoidosa* in both long-term growth tests and in short-term tests of ability to assimilate carbon dioxide in the light. Under conditions of growth inhibition bacitracin, erythromycin, and oxytetracycline appeared to have some stimulatory effect on carbonate-fixation. Gliotoxin, thiolutin, polymyxin B, and streptomycin inhibited the carbonate-fixation.

For the latter three compounds, a study by chromatography-autoradiography provided some evidence that the carbonate-fixation inhibitions were consequences of other, more direct inhibitions.

The authors are indebted to Miss Linda Simpson for the radioactivity measurements.

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STUDIES ON THE GROWTH OF CITRUS SEEDLINGS WITH DIFFERENT FORMS OF NITROGEN IN SOLUTION CULTURES[1]

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Citrus has been grown commercially with practically every known source of N. Comparative studies with different sources of N are few (6, 8, 11, 13, 15), but as a whole they have not indicated any preference by citrus for ammoniacal (NH_4) or nitrate (NO_3) N except in extremely acid soils (6, 8). To what extent citrus can utilize NH_4 directly cannot be judged from field tests because under conditions favorable for growth nitrification proceeds rapidly and NO_3 is formed regardless of what material is used as a source of N. The use of NH_4 in extremely acid soils is generally regarded as a poor practice for citrus, but no unanimity exists as to the reason. One of the usual explanations is that NH_4 is toxic to citrus roots (6, 11).

A review (7) of nitrogen studies with plants in general shows that NH_4 nutrition differs from NO_3 nutrition in three main ways; (a) the demand for oxygen to the roots is increased with NH_4 nutrition, (b) competition for absorption of cations other than NH_4 may be detrimental to growth if the supply of other cations is low, and (c) there may be indirect effects associated with shifts in pH of the medium such as the availability of Mo and P at low pH resulting from use of NH_4 and of heavy metals such as Mn and Fe at high pH.

The studies with citrus in artificial cultures (4, 6, 11) have not been sufficiently critical to establish whether plants of this genus can use NH_4 as an exclusive source of N. Haas (4) was able to maintain growth of citrus in solution cultures for an extended period with NH_4, but his results might be questioned since he assumed that nitrification did not take place in this type of culture. It is also difficult to explain why he found better growth in highly acidic solutions (pH 4.0 to 4.5) than in less acidic ones (pH 6.0 to 6.5). Van der Merwe (6), on the other hand, found NH_4 very detrimental to the growth of citrus in both sand and water cultures, but he apparently did not give adequate attention to pH control or aeration. That citrus roots can absorb NH_4 readily was clearly demonstrated by the studies of Bitcover and Wander (2) and Wallace (16), but the consequence of continued feeding with this form of N was not demonstrated.

In 1951 a series of experiments was started with the main objective of ascertaining whether citrus could grow normally with NH_4 as an exclusive source of N. Soil, sand and solution cultures were all tried as methods of culture, but the only method that permitted control of the several factors involved was the last. Consequently, the results presented are mostly from solution culture studies.

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MATERIALS AND METHODS

Preliminary solution culture tests of different ratios of NO_3 : NH_4 on young seedlings of six different kinds of citrus were made. These were Rough lemon, Sunki mandarin, Cleopatra mandarin, sour orange, grapefruit, and Pineapple sweet orange. In the absence of pH adjustment, these all behaved similarly; good initial growth occurred in all cultures, but later there were reduced growth and death of roots when the proportion of NH_4 was high. This response was always accompanied by development of extreme acidity (pH as low as 2.4). For continued work, the Pineapple sweet orange was chosen as a test plant except for one test in large outdoor sand cultures where commercial nursery-sized Valencia orange trees budded on Cleopatra mandarin stock were used.

Solutions were made from C.P. salts supplied in the following concentration of essential elements (ppm): N 100, P 10, K 40, Ca 80, Mg 36, Mn 2, Fe 1, Zn 0.05, Cu 0.01; B 0.25 and Mo 0.001. Fe was supplied as Fe-EDTA. All solutions contained a minimum of 5 ppm of SO_4, but the amount was much greater in the solutions containing NH_4 as this form of N came entirely from $(NH_4)_2SO_4$.

The solution cultures were in 3-gallon glazed, clay crocks continuously aerated by a stream of air that agitated the solution fairly vigorously. The seedlings were suspended in each crock by plywood lids painted with plastic. Newly emerged seedlings with the first two leaves only and with cotyledons removed were used. At this stage dried seedlings weighed less than 0.25 gm each.

Commercial dolomitic lime was used to counteract acidity in most cases. This was screened through a 20-mesh screen; the finest particles washed out by allowing a stream of tap water to overflow a large beaker containing the dolomite for about 1 hour; and then dried and sterilized at 100° C.

Tests for nitrification using brucine as an indicator (9) were made at intervals. Various minerals in leaves were determined by previously indicated methods (11).

RESULTS AND DISCUSSION

Studies with pots of soil are not discussed because the results are virtually identical with those recently described for field trials (13). The nursery-size trees in 55-gallon sand cultures showed results practically identical with those described by van der Merwe (6). Extremely poor growth was made in both half-NH_4 and all-NH_4 cultures. In these cultures the pH was below 4 most of the time regardless of the pH of the applied solution (4.5 and 6.5). At the end

of 1 year, these trees were about one-fourth the size of trees receiving all-NO_3, and they showed severe leaf symptoms of Mo deficiency (14). Repeated use of foliage sprays containing Mo resulted in improved appearance of foliage, but little additional growth was made and the root condition remained very poor; growth was very stubby and numerous roots were dead. Excellent growth was finally achieved after removing the top 6 inches of sand and mixing it with dolomitic lime at the rate of 1 lb of lime per 500 lb of sand and replacing it in the containers. However, no attempt was made to follow nitrification, and therefore detailed growth measurements are not given.

GROWTH STUDIES WITH DIFFERENT RATIOS OF NO_3 : NH_4 IN SOLUTION: In February 1953, duplicate 3-gallon cultures containing NO_3 and NH_4 in the 6 ratios shown in table I were started. These were maintained for 15 months, fresh solutions being made monthly. Excellent and essentially equal growth was made in all cultures. The study was terminated because the plants had outgrown the containers and because it seemed to be amply demonstrated that sweet orange seedlings would thrive under these conditions.

Control of acidity was achieved in the early months by adding NaOH twice weekly to the high-NH_4 cultures and H_2SO_4 to the all-NO_3 cultures to bring the pH to about 6. The volume of solution was relatively large in proportion to the amount of roots during this period and the shifts in pH were very gradual. However, as the plants got larger the shifts, particularly the drop in pH in the high-NH_4 cultures, were much more rapid. It was found that this acidity development could be very effectively prevented by putting about 10 gm of dolomitic lime in each crock each month. With this technique, a continuous pH of about 6.2 was maintained for the last 9 months of the test in all cultures except the all-NO_3 which tended to become alkaline (pH about 7.5). Occasionally H_2SO_4 was added to the all-NO_3 cultures to bring the pH down to about 5, but no attempt was made to hold the pH constant.

TABLE I

GROWTH MEASUREMENTS OF AERIAL PARTS OF PINEAPPLE ORANGE SEEDLINGS GROWN FOR 15 MONTHS IN COMPLETE NUTRIENT SOLUTION WITH DIFFERENT PROPORTIONS OF AMMONIACAL NITROGEN

TREATMENT	STEM HT	CROWN DIAM	LEAVES	DRY LF WT
	cm	*mm*	*no./plant*	*gm/plant*
All-NO_3	95	12.8	205	26
¼-NH_4	86	11.7	175	25
½-NH_4	85	11.1	170	24
¾-NH_4	90	11.3	205	29
⅞-NH_4	95	11.3	190	27
All-NH_4	94	11.8	166	29
F-value *	N.S.	N.S.	N.S.	N.S.

* Statistical symbols used in tables I to IV: N.S. = difference not significant at odds of 19 : 1. L.S.D. = least significant difference required between two means at odds of 19 : 1.

TABLE II

MEAN WEIGHT OF PINEAPPLE ORANGE SEEDLINGS AFTER 4 MONTHS GROWTH IN SOLUTION CULTURES WITH DIFFERENT FORMS OF NITROGEN AND WITH pH HELD NEAR 6.0

TREATMENT	pH RANGE	DRY WT PER PLANT (GM)			
		LEAVES	STEMS	ROOTS	TOTAL
¾-NO_3 + ¼-NH_4	6.2–5.8	4.48	2.12	4.15	10.75
¾-NO_3 + Dolomite	6.4–6.3	4.79	2.08	4.30	11.17
$(NH_4)_2SO_4$ + Dolomite	6.3–6.1	5.08	2.18	3.75	11.01
$(NH_4)_2CO_3$ + Dolomite	6.3–6.1	4.51	2.01	3.86	10.38
F-value	...	N.S.	N.S.	N.S.	N.S.

Tests for Ca and Mg in the nutrient solutions showed sufficient quantities of these elements were supplied by the solubilization of dolomite in the all-NH_4 cultures so that none need be added as soluble salts. The cultures were operated this way for the last 6 months.

These results (table I) strongly suggest that at least under certain conditions, citrus can utilize NH_4 as a sole source of N. However, tests made during the course of the experiment showed that nitrification occurred readily in the solution cultures. Only trace amounts of NO_3 could be found in the NH_4 cultures during the first 2 weeks after new solutions were applied. However, after 4 weeks there would typically be no NH_4 remaining, but 10 to 15 ppm of N as NO_3 was present. The oxidation of NH_4 was subsequently found to occur when either NaOH or dolomite was used for pH adjustment. Furthermore, about 1 % of the N in the C.P. $(NH_4)_2SO_4$ was found to be present as NO_3. Thus, it is not possible to conclude that any of the trees in this experiment lived exclusively on NH_4, even though it was the predominant form of N.

GROWTH IN THE ABSENCE OF NO_3: A subsequent experiment was carried out for a shorter period using the same technique as in the experiment just discussed but only the 3/4-NO_3 and the all-NH_4 treatments were compared in quadruplicate. In this test, the trace of NO_3 in the $(NH_4)_2SO_4$ salt was avoided in one treatment by neutralizing $(NH_4)_2CO_3$ with H_2SO_4. The solutions were renewed every 10 days for a 4-month period. Under these conditions nitrification never produced more than a trace of NO_3, and the all-NH_4 cultures were high in NH_4 at all times. The results are shown in table II.

Growth was also excellent in all of these cultures. The tops of the seedlings were dark green and entirely free of deficiency symptoms. Observations, as well as the dry weight data (table II), suggest that slightly greater root development occurred in the presence of NO_3; but the difference was not significant statistically. The roots in the NH_4 cultures were straight and smooth with no indication of any toxic effect of this form of N.

GROWTH IN RELATION TO pH AND N SOURCE: Since NH_4 sources of N are characteristically acidu-

lating to the substrates in which plants grow, two series of cultures were set up in triplicate at the pH ranges indicated in table III. The test ran for a 7-month period from late October 1954 to early June 1955. The solutions were changed monthly and no attempt was made to avoid nitrification. It was noted, however, that no nitrification took place in the acid range, while appreciable NO_3 (5 to 15 ppm) had developed in the high-pH NH_4 cultures tested each month just prior to solution change.

The pH ranges indicate the usual starting pH and the extreme deviation found during the test. Dolomite was supplied in smaller quantities (2 gm per 3-gallon crock) than previously used in the NH_4 cultures, and it was found that toward the end of the test this was not sufficient to prevent a drop in the pH over a month's period. Actually, the pH was above 6 for all but a few days during the entire 7-month period. The all-NO_3 cultures were started at pH 5.5 and allowed to drift upward. Weekly they were returned to 5.5 by adding H_2SO_4. The low-pH cultures were adjusted weekly to the indicated starting pH with either NaOH or H_2SO_4, depending on the direction of shift. Actually, the solutions with ½ or all-NH_4 were below 4.0 and the all-NO_3 above 4.0 most of the time. The downward drift in the NH_4 solutions was substantially reduced during the last 3 months of the test by adding 15 gm of coarse dolomite gravel (about 2-mm diameter) to each crock. Dissolution of these particles greatly slowed down the rate of acidulation of the solution but did not entirely prevent it.

While overall growth was essentially equal regardless of N source in cultures kept at the high-pH range, greater root development occurred in the all-NO_3 ones (table III), substantiating the trend shown in table II. The roots in the all-NO_3 cultures tended to be long, wiry and unbranched. This characteristic made them appear much more voluminous than the shorter, denser root systems in the NH_4 cultures (fig. 1). The older portions of the long NO_3 roots had considerable secondary thickening and wood development which probably was largely responsible for their greater dry weight.

TABLE III

MEAN WEIGHT OF PINEAPPLE ORANGE SEEDLINGS AFTER 7 MONTHS IN SOLUTION CULTURES WITH DIFFERENT PROPORTIONS OF AMMONIACAL NITROGEN AND DIFFERENT pH RANGES

N SUPPLIED AS	pH RANGE	DRY WT PER PLANT (GM)			
		LEAVES	STEMS	ROOTS	TOTAL
All-NO_3	5.5–6.9	9.1	5.7	7.6	22.4
½-NH_4	6.3–5.0	10.5	6.1	6.7	23.2
All-NH_4	6.3–4.5	9.9	5.1	6.2	21.2
All-NO_3	3.8–5.1	6.8	3.9	4.6	15.3
½-NH_4	4.1–3.5	6.6	2.9	3.6	13.1
All-NH_4	4.1–3.4	5.2	2.2	2.5	9.9
L.S.D. @ 0.05	...	1.5	1.2	1.3	2.3

TABLE IV

CONCENTRATION OF MACRO-ELEMENTS AND TOTAL BASE EQUIVALENTS IN DRIED LEAVES OF PINEAPPLE ORANGE SEEDLINGS GROWN FOR 7 MONTHS IN SOLUTIONS CONTAINING DIFFERENT NO_3 : NH_4 RATIOS AT DIFFERENT pH RANGES

N SUPPLIED AS	pH RANGE	PERCENT IN DRY LEAVES					TOTAL BASES *
		N	P	K	Ca	Mg	
All-NO_3	5.5–6.9	2.6	.26	2.01	2.48	.52	219
½-NH_4	6.3–5.0	2.8	.26	1.91	1.65	.47	170
All-NH_4	6.3–4.5	3.3	.30	1.83	1.49	.52	164
All-NO_3	3.8–5.1	2.5	.22	1.83	1.77	.39	166
½-NH_4	4.1–3.5	3.1	.25	1.77	1.61	.43	160
All-NH_4	4.1–3.4	3.6	.28	1.70	1.20	.49	144
L.S.D. @ 0.05	...	0.4	.04	0.08	0.12	.06	5

* Total K, Ca, Mg as meq/100 gm dried leaves.

Growth in the all-NH_4 cultures with the pH near 4 was poor. The foliage was normal in appearance but reduced in quantity. Root growth was severely reduced and the laterals were stubby and occasionally had swollen root tips. Growth was reduced also in the all-NO_3 cultures repeatedly acidified to near pH 4 but not as markedly as in the all-NH_4 cultures. In all probability the difference between these two would have been reduced if it had been possible to maintain a more nearly equal pH status. In solutions as weakly buffered as these, the H-ion concentration would have been several-fold greater most of the time in the all-NH_4 cultures. The plants on ½-NH_4 were intermediate in growth, and these cultures were appreciably slower in generating extreme acidity than were the all-NH_4.

At the termination of the culture period the leaves were analyzed for 10 elements. Both mature and immature leaves were included. The results for the primary elements are shown in table IV. Higher levels of N and P were found in relation to the proportion of NH_4 supplied and the trends were essentially the same at both pH ranges. Cation content was affected by both NH_4 and H, resulting in a net reduction in total bases when expressed as meq per 100 gm dry leaf matter. However, NH_4 depressed only K and Ca while H depressed K, Ca and Mg. The depressive effects of NH_4 and H on K and Ca appear to be additive as the greatest total base reduction occurred when these factors were working together.

Analyses for secondary elements (not presented) showed no relation between treatment and leaf concentration of B, Cu, or Mn while Fe and Zn were substantially lower in the all-NO_3 treatments. These elements were well above the deficiency levels, however, in all cases (10).

GENERAL DISCUSSION

The nutrient solutions used in the present studies were relatively dilute in comparison with most other culture solutions. Various tests over a period of years

have shown no advantage from high levels of the macro-nutrient elements for the growth of citrus. In fact, the management of cultures is easier where the levels of K and P are reduced as there is less tendency for unbalanced nutrition and induced deficiency symptoms. Leaf composition of plants grown with the levels used here have a mineral pattern very similar to that of healthy, field-grown trees when leaves of similar ages are compared.

It is probable that the use of dilute solutions tends to minimize some of the indirect factors involved in a study of the nutrition of different forms of N. For example, Chapman et al (see 7) found that high-PO_4 interferes with NO_3 absorption by citrus and high-NO_3 levels are necessary to offset this. High-PO_4 also complicates the nutrition of the heavy metals and frequently leads to chloroses due to inadequate utilization of metals. While critical studies have shown that most plants can readily use NH_4 (7), contrary results may be due in part to the lack of consideration of the high-SO_4 which accompanies a high supply of N from $(NH_4)_2SO_4$. Aldrich et al (1) noted a marked suppression of growth in lemons when SO_4 was high.

The present results leave little doubt that citrus can make healthy growth under suitable conditions when NH_4 is the source of N. Contrary results (4, 6, 11) probably resulted primarily from the indirect effects of excess acidity. Relative unbalance of other nutrients could also have been contributing factors as Haas (4) used 52 ppm PO_4 (rather high) and 21 ppm K (rather low). Arnon (see 7) found with barley that NH_4 accentuated the uptake of PO_4 and depressed the uptake of K. Others (7) have substantiated this with different plants. The present results with citrus also are in agreement with Arnon's results in this respect.

Guest and Chapman (3) attempted to ascertain the relation of pH to the growth of citrus over a very wide range (2.0 to 11.0). Numerous difficulties were encountered with indirect factors and the results were not clear-cut. They assert, however, that there is no appreciable direct effect of pH from slightly below 4 to somewhat above 9. From the results of miscellaneous tests, Haas (4) reported optimal growth of citrus at pH 4.0 to 4.5 and very poor growth above pH 6.0. Both of these reports, however, describe attending toxicities or deficiencies of heavy metals. The present results, while not attempting to establish the optimal pH for growth, showed a definite suppression of growth at pH's near 4, as contrasted to pH's near 6, irrespective of N form.

Leaf analysis indicates that cation absorption is interfered with by both NH_4 and H and the individ-

FIG. 1. Seven-month-old Pineapple orange seedlings grown with N supplied as NH_4 (*left*) and NO_3 (*right*) in cultures maintained at pH's near 4 and 6. Background lines are 4 inches apart.

ual suppressions tend to be additive. It seems probable, however, that no cause and effect relation exists between the suppression in base absorption and the reduced growth. Leaf K and Mg levels were all in the luxury range and the depressions noted for them would not likely suppress growth. Ca suppression is more likely related to the depression in growth, but even the leaf content of this element was greater than that previously associated with critical deficiency in citrus (10). It seems most likely that the unfavorable effect of low pH is somehow attributable to H-ion and the accentuated effect of NH_4 at low pH is related to the greater acidity generated by NH_4 absorption. The various studies by Arnon and associates (see 5) showed general lethal effects at pH 3 with various plants grown in culture solutions. Reduced growth at pH 4 could be only slightly alleviated by increased Ca supplies and normal growth was never attained at pH's as low as 4, irrespective of solution composition. The present results with citrus seedlings seem to be in harmony with these previous studies with annual plants.

So far as the author is aware, this is the first description of a method of continuously neutralizing acidity in culture solution and not unduly raising the pH through the use of relatively insoluble carbonates.

Summary

Freshly emerged Pineapple sweet orange seedlings were successfully grown for periods up to 15 months in aerated solutions with N supplied as $(NH_4)_2SO_4$. Such plants were healthy and essentially equal to others grown with all or part of their N supply from NO_3 provided the development of excess acidity was prevented. Somewhat greater root development occurred when NO_3 was the source of N.

When the solution pH was maintained near 4.0, growth was reduced irrespective of form of N. Greater reduction in growth occurred in the NH_4 cultures than in the NO_3 cultures, however, greater acidity also existed due to the opposite trends in pH shifts as a result of absorption.

The reduction in growth was paralleled by a reduced concentration of base cations in the foliage. Reduced K, Ca and Mg concentrations were associated with increased H-ion concentration, while the shift from NO_3 to NH_4 reduced the K and Ca only. It seems probable that these effects are largely coincidental with and not the primary cause of reduced growth under high acid conditions.

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GROWTH REPRESSION OF HIGHER PLANTS BY 2-PYRIDINETHIOL, 1-OXIDE[1,2]

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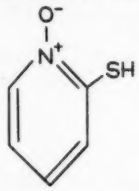
The compound 2-pyridinethiol, 1-oxide and its salts have been reported to have pronounced antibacterial and antifungal activity of a type that, were it a microbiological product, would cause it to be classed as a broad-spectrum antibiotic (5, 8). This property is shared by a number of other cyclic thiohydroxamic acids, variously substituted, though some are much more active than others. The high potency of this grouping, and the diversity of microorganisms to which compounds containing it are inhibitory, suggest interference with some essential metabolic process. Furthermore, certain antibiotics of comparable potency have been found to repress growth in some plant systems (1, 3, 4). Accordingly, the activity of 2-pyridinethiol, 1-oxide (PTO) and derivatives was tested on plants in various ways.[3]



METHODS

Most of the procedures followed are well established in practice and not critical in detail. Those found of greatest use in quantitative comparisons were (1) a modification of the bioassay method of Ready and Grant (7) whereby the repression of elongation of the primary root of cucumber seedlings, var. Early Fortune, is determined at 25° C and 96 hours and (2) the determination of repression of weight increase in roots of young barley seedlings, var. Moore or Atlas 46, in aerated deep culture at 25° C for 6 days in the dark (2). This is essentially a study of the effect of the test compound on the conversion of endosperm to roots.

RESULTS AND DISCUSSION

Experimentation with these compounds has largely been confined to root presentation studies because it was established initially that neither droplet application of 50 or 100 μgm PTO to a unifoliate leaf of a young bean plant nor 24-hour single leaf dipping experiments involving exposure of young plants to solutions of 10^{-4} or 10^{-3} M PTO caused any visible effect on subsequent top growth, growth habit or growth sequence. At the higher rates there was usually some contact injury to the tissue treated in summer temperatures. Later experiments involving exposure of the whole foliage of established seedlings did, however, result in development of some of the responses described below, but only at relatively high treatment rates.

Plant roots proved to be quite responsive to 2-pyridinethiol, 1-oxide presented as the sodium salt. Increase in root weight of barley seedlings was extensively inhibited by concentrations of only 3 to 5 μgm/ml (fig 1). The concentration effecting 50 % inhibition in root growth of Atlas barley at 25° C in 6 days was only 2.7 μgm/ml (1.8×10^{-5} M). Root elongation was similarly repressed. With cucumber as the test seedling even lower concentrations caused inhibition. The concentration causing 50 % reduction in length was only 1.4 μgm/ml or 9.5×10^{-6} M. These concentrations are in the same range as those found inhibitory to a number of pathogenic bacteria.

Eight analogs or derivatives of PTO prepared by the Squibb Institute were similarly subjected to the cucumber test. All had activity, but their potency

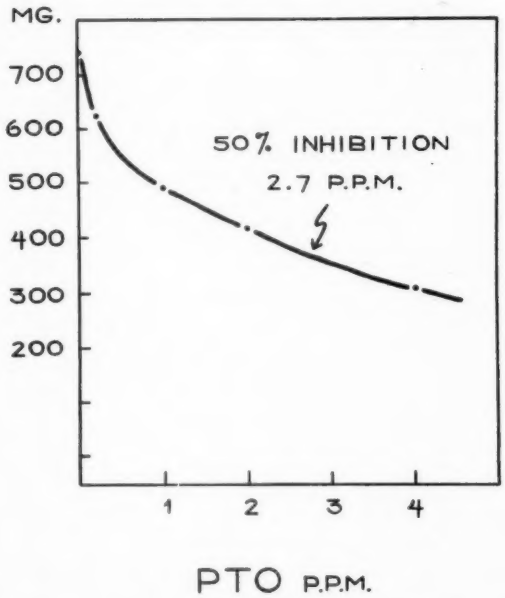


FIG. 1. Repression of root growth of barley seedlings treated with 2-pyridinethiol, 1-oxide, Na salt. Seedlings grown for 6 days at 25° C in the dark.

[1] Received July 3, 1956.

[2] Paper No. 12 from the Plant Nutrition Laboratory, Michigan Memorial Phoenix Project No. 32 of the University of Michigan.

[3] These compounds were supplied by the Squibb Institute for Medical Research through the courtesy of Dr. A. F. Langlykke. The compound 2-pyridinethiol, 1-oxide is under test as a fungicide and has been given the name Omadine.

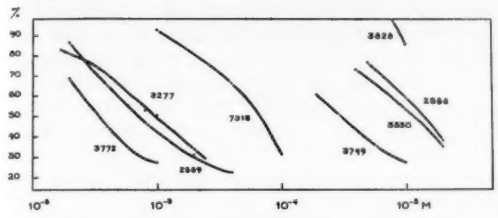


FIG. 2. Repression of elongation of roots of cucumber seedlings by analogs or derivatives of 2-pyridinethiol, 1-oxide. (For identification of the compounds, see table I.) Seeds incubated at 25° C for 96 hrs in the dark.

varied by several orders of magnitude (fig 2). It is interesting and perhaps of some significance that the three having the most pronounced antibacterial and antifungal activity were also those most active in repressing root elongation (table I). Below this level, however, the correlation was not perfect, and the next two on the list, 2-benzylmercaptopyridine, 1-oxide and 2-mercaptoisonicotinamide, 1-oxide, were relatively more inhibitory in the plant system than to microorganisms.

However, this does not weaken the general observation that the most active compounds of this type inhibit root growth and proliferation of microorganisms at approximately the same concentrations. No suggestion as to mode of action can yet be made; reversal studies have not been informative.

Numerous experiments were next carried out involving applications of PTO or dithiodipyridinedioxide (DTPO) to the roots of various plants. The plants were grown in vermiculite in quart plastic containers, and were supplied throughout with adequate nutrients. Applications of the chemicals were made in solution to well-established seedlings of various species, and their subsequent development carefully watched. The species included bushbean, soybean, tomato, sunflower, cucumber, corn, cress, flax, garden pea and petunia. In every case relatively low rates of application (5 to 15 mg/pot) resulted in the development of smaller or dwarfed plants. Higher rates were lethal, but the lethal thresholds were not determined. There were some differences between species in responsiveness to these compounds. Cucumbers, for example, were more sensitive than bushbeans or tomatoes. Representative series are shown in figure 3. Plants treated with sub-lethal levels did not exhibit any morphological abnormalities, nor in general was there any delay in flowering or alteration in flower development and seed set. The plants were smaller in height and leaf areas, with shorter internodes. Shortly after the application of PTO or DTPO and for some days thereafter, treated plants were observed to wilt if exposed to high light–high temperature–low humidity conditions, but if the soil moisture supply was adequate the wilt did not proceed to the point of irreversible leaf injury. Axillary bud development in decapitated plants was not affected by PTO application to the roots. Seeds harvested from bean plants dwarfed by application of PTO or DTPO in amounts up to 30 mg in the seedling stage germinated normally and in turn provided seedlings that were not smaller than the controls. It may be inferred that these com-

TABLE I

REPRESSION OF CUCUMBER ROOT ELONGATION BY 2-PYRIDINETHIOL, 1-OXIDE AND DERIVATIVES

COMPOUND		CONC. FOR 50 % REPRESSION		ANTIBACTERIAL ACTIVITY (MIC)*		ANTIFUNGAL ACTIVITY (MIC)*	
				MICROCOCCUS PYOGENES VAR. AUREUS	KLEBSIELLA PNEUMOCCUS	ASPERGILLUS FUMIGATUS	SACCHAROMYCES CEREVISIAE
		M	*μgm/ml*	*μgm/ml*	*μgm/ml*	*μgm/ml*	*μgm/ml*
MC 3772	2,2′-Dithiodipyridine-1, 1′-dioxide	3.4×10^{-6}	0.8	0.08	2	0.4	0.3
2589	2-Guanylmercaptopyridine, 1-oxide, hydrobromide	7.0×10^{-6}	1.7	0.2	3	0.8	0.3
3277	2-Pyridinethiol, 1-oxide, sodium salt	9.5×10^{-6}	1.4	0.03	1.5	0.4	0.4
7415	2-Benzylmercaptopyridine, 1-oxide	6.6×10^{-5}	14	> 100	> 100	> 25	> 25
3749	2-Mercaptoisonicotinamide, 1-oxide	3.0×10^{-4}	51	60	> 60	> 25	> 25
3830	4-Mercaptopyridine, 1-oxide	1.2×10^{-3}	146	15	> 60	> 13	> 13
2588	2-Pyridinethiol	1.4×10^{-3}	155	10	150	> 25	> 25
3828	2,2′-Thiodipyridine-1, 1′-dioxide	$> 1 \times 10^{-3}$	> 220	> 60	> 60	> 13	> 15
3863	2-(Pyridyl-1-oxide) mercaptoacetic acid	much $> 8 \times 10^{-3}$	> 1480	> 60	> 60	> 13	> 13

* Code number and minimum inhibitory concentration (MIC) data supplied by the Squibb Institute.

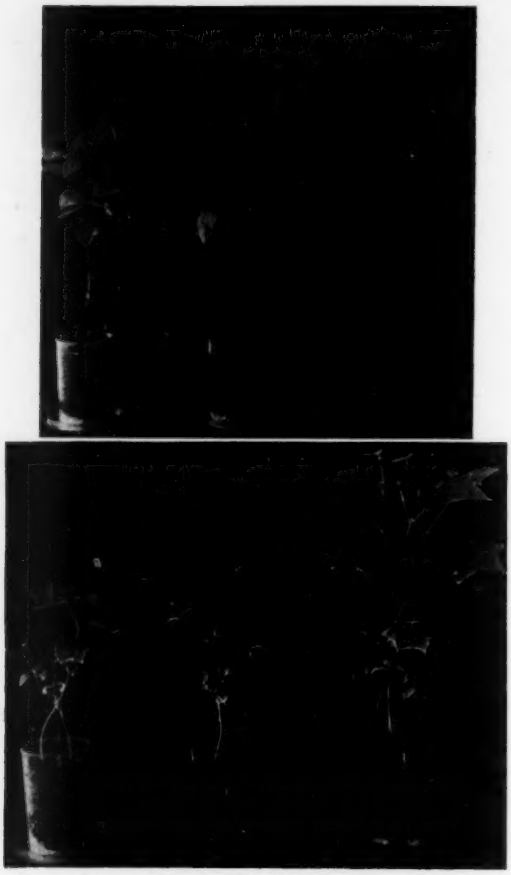


FIG. 3. Plants in vermiculite 30 days after treatment with 2-pyridinethiol, 1-oxide to 8-day-old seedlings. *Upper group.* Beans, 30 mg (left), 15 mg (center), untreated (right). *Lower group.* Cucumber, 15 mg (left), 7.5 mg (center), untreated (right).

pounds are not transferred to the seed in amounts sufficient to affect seedling growth, morphology or development.

Because in some situations maleic hydrazide (MH) or 1,2-dihydro-3,6-pyridazinedione acts as a growth repressant, comparisons were made of the effects of MH and PTO on seedlings of the same age. Maleic hydrazide causes retardation of the normal growth sequence; frequently also grave morphological aberrations are induced when growth is resumed. Neither effect has been seen in plants treated with PTO or DTPO.

Heavy applications (100 to 300 mg PTO) were made to large well-established tomato plants in soil, and to stocks and petunias (15 to 45 mg PTO) just prior to flower formation. In no case was there any discernible effect on number, size or time of flowering. This raised a question as to possible absorption of PTO by soil or its rapid inactivation by soil microorganisms. The latter seemed unlikely in view of the antifungal and antibacterial activity of this compound. Known concentrations of PTO were added to soil samples (10 ml 1×10^{-2} M and downwards to 35 gm air dry soil), and incubated for various periods. The samples were transferred to a Waring blendor with 500 ml water and blended for 30 seconds. Aliquots were centrifuged and subjected to bioassay using the cucumber root elongation test (7). It had previously been established that PTO concentrations of 1 μgm/ml could be readily determined. However, neither immediately nor after incubation was it found possible to elute from soil sufficient PTO to cause any significant repression of root elongation even though the initial quantity applied to the soil was as high as 14.9 μgm/35 gm soil. PTO, therefore, is quickly adsorbed and firmly retained by soil colloids. DTPO was found to behave similarly.

When added to soil, and presumably absorbed, PTO is not completely inactivated with respect to roots present in the soil, but substantially larger quantities have to be added to obtain the same degree of plant response as is elicited in quartz sand or vermiculite. Even in vermiculite, however, some adsorption appears to take place. In a comparative experiment, using beans in quart containers approximately the same extent of growth repression or dwarfing was obtained by the addition of 10 mg PTO to quartz, 40 mg to vermiculite, and 100 mg to soil.

From these varied experiments it is clear that PTO and some related compounds are potent in affecting the growth of some higher plants, though they cannot be regarded as growth-regulators in the sense in which this term is applied to the substituted phenoxyacetic acids. It appears that their growth repressant effect is manifest mainly, if not exclusively, in root development and root function, and that the dwarfed tops are the resultant of the limitation of the root system.

SUMMARY

2-Pyridinethiol, 1-oxide and certain of its derivatives, which possess broad antibacterial and antifungal activity, have been found to be inhibitory also in certain plant systems. Root elongation and dry matter increase in cucumber and barley seedlings are repressed at low concentrations, but applications of similar concentrations to the tops of several species have not resulted in discernible responses in top growth or development. Root presentation, however, can cause a generalized dwarfing of the plant without the appearance of morphological abnormalities. Recently treated plants may wilt under high moisture stress. The normal developmental sequence is not significantly delayed. Seed from treated plants gives normal seedlings. Higher application rates are lethal. 2-Pyridinethiol, 1-oxide is strongly adsorbed by soil; the dwarfing response, which appears to arise from interference with root development and function, can best be seen in plants grown in sand or vermiculite.

The author wishes to acknowledge the able assistance of Mr. Leo Vander Beek in many of these experiments.

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EFFECT OF CHEMICAL STRUCTURE ON THE GROWTH INHIBITION OF PLANTS WITH SOME ACID ANALOGS OF 2-MERCAPTOBENZIMIDAZOLE[1,2,3,4]

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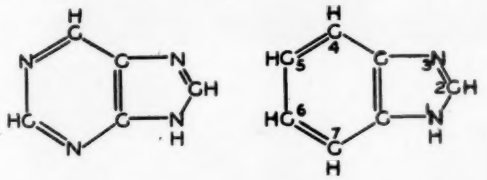
Several substances structurally related to benzimidazole occur naturally in biological systems. A derivative of this compound, 5,6-dimethylbenzimidazole, constitutes part of the vitamin B_{12} molecule. Purines, an important component of nucleic acid structures and nucleotides, bear a structural similarity to benzimidazole. Nucleic acids play an important role in growth and appear to be associated with the synthesis of proteins by living material. Nucleotides are required for many of the metabolic processes of plants and animals. Therefore, it would appear logical that substances which might act as antagonists of purine compounds would also influence the growth of plants.



PURINE BENZIMIDAZOLE

Several workers have indicated that benzimidazole is an antagonist of purine compounds. Woolley (5) noted that benzimidazole inhibited the growth of several yeasts and bacteria and observed that the inhibition could be completely removed by aminopurines. Klotz and Mellody (3) reported that yeast nucleic acid caused a reversal of the inhibitory effect of benzimidazole on the growth of the bacterium, *Escherichia coli*. Gillespie et al (2) demonstrated that 4-methoxy-6-methylbenzimidazole was an effective growth inhibitor of *Tetrahymena gelii*, a guanine-requiring protozoan, and also of developing embryos of the frog, *Rana pipiens*. By using peas as the test plant, Galston et al (1) found that benzimidazole was a metabolic antagonist of adenine and caused an inhibition of cell elongation. Recently Rebstock et al (4) demonstrated that several acid analogs of 2-mercaptobenzimidazole inhibited the growth of Cranberry bean plants and root formation of cucumber seeds.

In the present investigation the effect of chemical structure of a number of derivatives of 2-mercaptobenzimidazole on the growth inhibition of Cranberry bean plants and root development in cucumber seedlings was studied. Different chemical groups were substituted in the benzene ring of the benzimidazole nucleus and in the side chain. These compounds were synthesized in this laboratory and their preparation will be reported elsewhere.

Materials and Methods

Two methods of biological assay were employed for the evaluation of the inhibitory effects of the benzimidazole compounds upon the growth of plants.

One of the assays was the bean leaf test. Seeds of the bean plant (*Phaseolus vulgaris* var. Cranberry) were selected for uniformity of size and planted in four-inch pots in the greenhouse. After germination, all but the uniform seedlings were removed from each

[1] Received July 12, 1956.

[2] Journal Article No. 1934, Michigan Agricultural Experiment Station, East Lansing, Michigan.

[3] Supported in part by the Horace H. Rackham Research Endowment of Michigan State University.

[4] From the Ph.D. thesis of Theodore L. Rebstock, Michigan State University, 1956.

pot. Six to eight days after planting and at the expansion of the primary leaves, the plants were treated with an 0.005 M solution of the benzimidazole compound by dipping the expanding leaves into the test solution. Visual observations were noted in the relative amount of inhibition of new leaf and stem growth induced by each of the compounds as compared to control plants at a definite time interval after the treatments.

The solutions of the benzimidazole analogs were prepared by suspending enough of the compound in distilled water to give an 0.005 M solution. A definite volume of a saturated solution of sodium bicarbonate was added and the mixture stirred until solution was complete. The pH of the solution was then adjusted to 7.0 to 7.1 by the addition of an aqueous 25 % acetic acid solution and then the volume adjusted with distilled water. A solution of sodium bicarbonate neutralized with dilute acetic acid to pH 7 was used for the control plants.

Root inhibition of cucumber seeds (var. Marketer) was employed as an additional method of biological assay. Seeds were germinated on a filter paper impregnated with either a 0.005 or 0.0005 M solution of the test compounds in a Petri dish at room temperature (approximately 25° C) under laboratory conditions of alternating light and darkness. After eight days the longest root was measured and the length of this radical was used as an index of growth inhibition of the test compound as compared to control seedlings treated with a neutral solution of sodium bicarbonate and acetic acid.

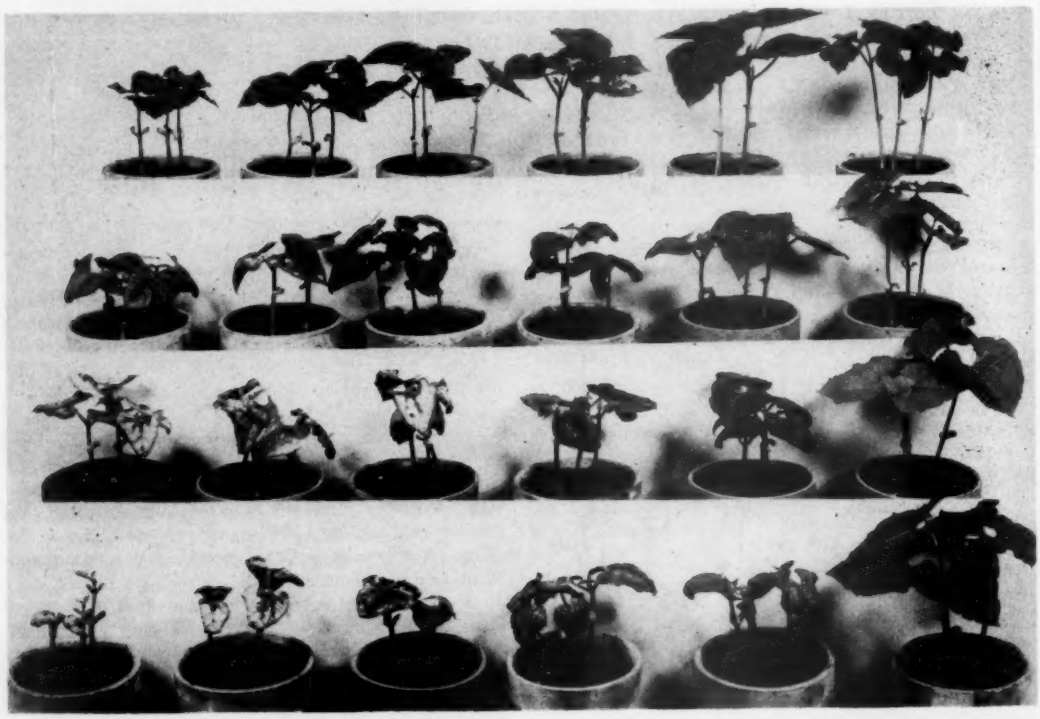


FIG. 1. The appearance of bean plants 5 days after treatment of the leaves with 0.005 M solutions of acid analogs of 2-mercaptobenzimidazole.

Row I (top, left to right). α(5-Phenyl-2-benzimidazolylthio)propionic acid. (5-Phenyl-2-benzimidazolylthio)-acetic acid. (4,5,6-Trichloro-2-benzimidazolylthio)acetic acid. α(5,6-Dimethyl-2-benzimidazolylthio)propionic acid. (5,6-Dimethoxy-2-benzimidazolylthio)acetic acid. Control.

Row II. (5-Methoxy-2-benzimidazolylthio)acetic acid. (5-Methyl-2-benzimidazolylthio)acetic acid. α(4,5,6-Trichloro-2-benzimidazolylthio)propionic acid. (2-Benzimidazolylthio)acetic acid. α(5-Nitro-2-benzimidazolylthio)-propionic acid. Control.

Row III. (5-Bromo-2-benzimidazolylthio)acetic acid. (5,6-Dichloro-2-benzimidazolylthio)acetic acid. β(2-benzimidazolylthio)propionic acid. α(5-Bromo-2-benzimidazolylthio)propionic acid. (4,6-Dichloro-2-benzimidazolylthio)acetic acid. Control.

Row IV (bottom, left to right). β(5-Chloro-2-benzimidazolylthio)propionic acid. (5-Chloro-2-benzimidazolylthio)acetic acid. α(2-Benzimidazolylthio)propionic acid. α(5,6-Dichloro-2-benzimidazolylthio)propionic acid. α(5-Chloro-2-benzimidazolylthio)propionic acid. Control.

Results and Discussion

When the bean leaf biological test was employed, the first effects of the compounds were clearly visible the day following the treatments. The leaves of the treated plants were curled and wrinkled and had acquired a deeper green color than those of the control plants. After two to three days the treated leaves of some of the plants became chlorotic. The appearance of typical plants five days after treatment with several of the compounds is illustrated in figure 1. The control plants are at the right of each row and are of the same age as the treated ones and grown under identical greenhouse conditions. The plants in each of the other pots were treated with a different derivative.

The figure clearly demonstrates that the response of the plants to each of the substances was not the same and that the compounds which caused the greatest inhibition of growth were the halogen substituted chemicals with the 5-chloro-analogs being the most effective.

Variations in the structure of the benzimidazoles had considerable influence on the inhibitory activity on bean plants. The β-propionic acids were slightly better inhibitors than the corresponding acetic acid derivatives, whereas the α-propionic acids were the poorest. As a rule, the acetic and β-propionic acid derivatives of the most active compounds caused a more pronounced chlorosis than did the α-propionic acid compounds.

The nature of the substituents in the aromatic nucleus affected the inhibitory activity of the 2-mercaptobenzimidazole analogs more than did the variations in the acid side chains. A chlorine or a bromine atom in the 5-position of the benzimidazole nucleus considerably increased the inhibitory action with a chlorine atom slightly more effective than a bromine atom. The substitution of a second chlorine atom in the 6-position yielded a compound which was a better inhibitor than the unsubstituted compound, but not quite as effective as the 5-monochlorobenzimidazole. Substitution of chlorine atoms in positions 4 and 6 reduced the inhibition to about that of the unsubstituted compound, namely (2-benzimidazolylthio)acetic acid. Three chlorines substituted in positions 4, 5, and 6 almost completely eliminated the inhibitory activity of the benzimidazole compound.

Nitro-, phenyl-, methyl-, and methoxyl-groups appreciably decreased the inhibitory action and the substitution of two methyl groups in either the 5 and 6 or the 4 and 6-positions almost completely destroyed the biological activity of these compounds.

The inhibition of new leaf and stem growth was also used as a qualitative index to evaluate the growth activity of each compound as compared to control plants (table I).

Generally, the compounds which caused the most inhibition of growth of bean plants also caused the greatest inhibition of root formation of cucumber seeds. The radicles which did emerge from the treated seeds were almost devoid of root hairs, whereas the radicles from the control seeds had numerous root hairs. In almost all instances, the degree of inhibition was greater when the seeds were treated with a 0.005 M solution than it was with a 0.0005 M solution. However, with a few exceptions, the tenfold dilution of the test solution did not reduce the inhibitory activity of the α-propionic acid derivatives as greatly as it did the acetic acid compounds. Although, at the greater concentration the acetic acid derivatives were usually better inhibitors than the corresponding α-propionic acid compound, the β-propionic acid derivatives were slightly more inhibitory than the derivatives of the other acids.

Table I

Inhibitory Activity of Acid Analogs of 2-Mercaptobenzimidazole on the Growth of Cranberry Bean Plants

Acid	Relative inhibition *	Degree of chlorosis
(2-Benzimidazolylthio)acetic	+++	Slight
α(2-Benzimidazolylthio)propionic	+	Slight
β(2-Benzimidazolylthio)propionic	+++	Severe
(5-Chloro-2-benzimidazolylthio)-acetic	+++++	Severe
α(5-Chloro-2-benzimidazolylthio)-propionic	++	Moderate
β(5-Chloro-2-benzimidazolylthio)-propionic	++++++	Severe
(4,6-Dichloro-2-benzimidazolylthio)-acetic	++++	None
(4,5,6-Trichloro-2-benzimidazolylthio)acetic	0	Slight
α(4,5,6-Trichloro-2-benzimidazolylthio)propionic	+	None
(5-Bromo-2-benzimidazolylthio)-acetic	+++++	Severe
α(5-Bromo-2-benzimidazolylthio)-propionic	++	Slight
(5-Nitro-2-benzimidazolylthio)-acetic	0	None
α(5-Nitro-2-benzimidazolylthio)-propionic	+	None
(5-Methoxy-2-benzimidazolylthio)-acetic	+	Slight
α(5-Methoxy-2-benzimidazolylthio)-propionic	0	None
(5-Methyl-2-benzimidazolylthio)-acetic	+	Moderate
α(5-Methyl-2-benzimidazolylthio)-propionic	0	None
(4,6-Dimethyl-2-benzimidazolylthio)acetic	+	None
α(4,6-Dimethyl-2-benzimidazolylthio)propionic	+	None
(5-6-Dimethyl-2-benzimidazolylthio)acetic	0	None
α(5,6-Dimethyl-2-benzimidazolylthio) propionic	0	Slight
(5-Phenyl-2-benzimidazolylthio)-acetic	+	None
α(5-Phenyl-2-benzimidazolylthio)-propionic	+	None
Control plants	0	None

* The number of plus signs indicate the relative magnitude of inhibition of new leaf and stem growth as compared to control plants 8 days after treatment with a 0.005 M solution of the test compound.

TABLE II

RELATIVE ROOT LENGTH OF CUCUMBER SEEDLINGS 8 DAYS AFTER TREATMENT OF THE SEEDS WITH SOLUTIONS OF ACID ANALOGS OF 2-MERCAPTOBENZIMIDAZOLE *

ACID	CONCENTRATION	
	0.005 M	0.0005 M
(2-Benzimidazolylthio)acetic	74	69
α(2-Benzimidazolylthio)propionic	59	64
β(2-Benzimidazolylthio)propionic	51	55
(5-Chloro-2-benzimidazolylthio)-acetic	46	55
α(5-Chloro-2-benzimidazolylthio)-propionic	64	68
β(5-Chloro-2-benzimidazolylthio)-propionic	28	48
(4,6-Dichloro-2-benzimidazolylthio)-acetic	62	84
(5,6-Dichloro-2-benzimidazolylthio)-acetic	41	52
α(5,6-Dichloro-2-benzimidazolylthio)propionic	59	66
(4,5,6-Trichloro-2-benzimidazolylthio)acetic	54	88
α(4,5,6-Trichloro-2-benzimidazolylthio)propionic	62	64
(5-Bromo-2-benzimidazolylthio)-acetic	46	75
α(5-Bromo-2-benzimidazolylthio)-propionic	51	53
(5-Nitro-2-benzimidazolylthio)-acetic	59	66
α(5-Nitro-2-benzimidazolylthio)-propionic	67	90
(5-Methoxy-2-benzimidazolylthio)-acetic	64	84
α(5-Methoxy-2-benzimidazolylthio)-propionic	73	68
(5-Methyl-2-benzimidazolylthio)-acetic	49	79
α(5-Methyl-2-benzimidazolylthio)-propionic	64	157
(4,6-Dimethyl-2-benzimidazolylthio)acetic	46	88
α(4,6-Dimethyl-2-benzimidazolylthio)propionic	33	96
(5,6-Dimethyl-2-benzimidazolylthio)acetic	72	106
α(5,6-Dimethyl-2-benzimidazolylthio)propionic	59	106
(5-Phenyl-2-benzimidazolylthio)-acetic	31	71
α(5-Phenyl-2-benzimidazolylthio)-propionic	44	77
Control	100	100

* Results are expressed as the percentage of the root length of controls.

As was observed with the bean leaf test, the substitution of a halogen in the benzene nucleus of the benzimidazole resulted in an increase in inhibitory activity. The 5-chloro- and 5-bromo-compounds caused about the same degree of inhibition when tested at a 0.005 M concentration, but at the higher dilution, the 5-chloro-compounds were slightly more inhibitory. The 5,6-dichlorobenzimidazoles were almost as effective as the corresponding monohalogen compounds, whereas the 4,6-dichloro-derivative was less effective and caused about the same effect as did the acetic acid derivative of 4,5,6-trichloro-2-mercaptobenzimidazole.

The methyl substituted compounds markedly inhibited root formation at a concentration of 0.005 M but at the more dilute concentration the inhibition was almost negligible. In some cases, root formation may have been stimulated as was evident by the appearance of numerous secondary root hairs.

The 5-nitro-, 5-phenyl-, and 5-methoxyl-compounds inhibited the growth of the bean leaf to a slight degree but a different response was noted in the cucumber root test. The methoxyl- and nitro-compounds were among the least effective of the inhibitors tested, but the 5-phenyl-derivatives caused an appreciable inhibition at a concentration of 0.005 M and still some inhibition at a concentration of 0.0005 M. The results of the cucumber test are summarized in table II.

SUMMARY

1. A study was made of the effect of chemical structure of a number of acid analogs of 2-mercaptobenzimidazole on growth inhibition of Cranberry bean plants and root development in cucumber seedlings.

2. Variations in the structure of the benzimidazoles greatly influenced the growth inhibition. The β-propionic acids were slightly more inhibitory than the corresponding acetic acid derivatives whereas the α-propionic acids were usually less active.

3. A chlorine or a bromine atom in the 5-position of the benzimidazole nucleus greatly enhanced the inhibition with a chlorine atom slightly more effective than a bromine atom. The 5,6-dichloro-derivative was almost as effective as the 5-monochlorobenzimidazole derivative and the 4,6-dichloro-compound was of the same order of magnitude as the unsubstituted parent compound, (2-benzimidazolylthio)acetic acid. Three chlorine atoms in positions 4, 5 and 6 almost completely removed the growth inhibition of the benzimidazole compound.

4. Nitro-, phenyl-, methyl-, and methoxyl-groups greatly decreased the inhibitory activity and the substitution of two methyl groups in either the 5 and 6 or the 4 and 6 positions almost completely destroyed the ability of these compounds to inhibit the growth of the test plants.

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SOME EFFECTS OF ASCORBIC ACID AND METAL-COMPLEXING AGENTS ON THE RESPIRATION OF BARLEY ROOTS[1,2]

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That ascorbic acid oxidase supports salt accumulation by barley roots has been inferred from particular inhibitions of respiration and salt uptake and the assumption that cytochrome oxidase is non-functional or even absent from the roots of seedlings older than 6 days (9, 24). The latter assumption may be unwarranted because cytochrome oxidase has been isolated from roots of 10- to 12-day-old barley of different varieties (2, 7). Under certain conditions the uptake of potassium and bromide has been shown to depend upon cytochrome oxidase in barley roots of one variety (23). Thus, it is moot whether the quantitative role of an oxidase system in supporting physiological activity can be determined solely by inhibition of respiration (4, 10). In particular, the value of diethyldithiocarbamate in estimating the activity in vivo of ascorbic acid oxidase (12, 13) has been the subject of the experiments described here. The results indicate the difficulty of assessing the quantitative role of oxidases from respiratory inhibitions. Some aspects of the respiratory characteristics of barley roots were reexamined in order to present a comparison with previous reports.

METHODS AND MATERIALS

PREPARATION OF BARLEY: The roots of Cape barley (*Hordeum vulgare*) were used as experimental material. The seedlings of different ages were grown as previously described (7). If not otherwise stated, the 0 to 10 mm region was used, where the root tip is the zero point.

INHIBITOR TREATMENTS: The effects of metal-complexing agents were induced by treatment with the agents before the actual respiratory measurements, and also treatment during the respiratory measurements.

The diethyldithiocarbamate[4] pretreatment consisted of soaking 100 or 150 segments at room temperature for 15 minutes in three 10-ml volumes of 10^{-3} M DDC, each prepared 3 minutes before use in pH 5.8 buffer. Usually the buffer was 5.2×10^{-2} M TRIS plus 2.0×10^{-2} M EDTA. Exceptions are noted as required. Segments soaked in water or buffer were used as reference controls. After the pretreatment the segments were rinsed with three 10-ml volumes of buffer, and placed in Warburg vessels for respiratory measurements. Weights of the segments were determined prior to DDC pretreatment in these cases.

Pretreatment with phenylthiourea and 8-hydroxyquinoline consisted of soaking 100 root segments per 10 ml of the agents for 17 to 20 hours at 25° C. Segments soaked in water were reference controls. After soaking, the segments were rinsed with three portions of distilled water. The weights of the segments were determined and the respiratory activities were measured.

Cyanide inhibitions were determined with KOH as carbon dioxide absorbent and verified with 2 M KCN as absorbent (14).

RESPIRATORY MEASUREMENTS: The direct manometric method of Warburg was used to measure oxygen uptake at 25° C and Warburg's direct two-vessel method for carbon dioxide production. Respiration was measured with 100 or 150 root segments in 3 ml of water or buffer and buffer plus additions. For experiments with ascorbate, 0.2 ml of TRIS-neutralized ascorbate (8.93×10^{-2} M AA plus 8.8×10^{-2} M TRIS in buffer) were added to the reaction mixtures from the sidearm after a temperature equilibration of 15 minutes. The amount of ascorbate added was sufficient to reduce 200 μl of oxygen. Activities reported are generally uncorrected for autoxidation of ascorbate since autoxidation is very low in the TRIS-EDTA buffer. Corrections were applied when buffers other than TRIS-EDTA were used.

Activities were converted to Q_{O_2}(FW) or Q_{CO_2} (FW), based upon weights of the root segments. Prior to respiratory measurements ten samples of fifty segments were weighed with a torsion balance after excess liquid was removed with filter paper.

ENZYME EXTRACTIONS: Homogenates of the root segments in distilled water were the sources of the enzyme preparations. Preparations of the distilled water homogenates followed the procedure previously described (7). The homogenates were not filtered through muslin but were used directly in the fractionation procedure given in figure 1. All procedures were carried out in a cold room, $< 5°$ C, with ice-chilled materials. Centrifugations were carried out with a refrigerated centrifuge, -2 to 0° C. Estimates of activity recovery were based upon the number of segments homogenized and the equivalent numbers

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[4] Abbreviations used are: DDC, sodium diethyldithiocarbamate; PTU, phenylthiourea; 8-OH-Q, 8-hydroxyquinoline; TRIS, Tris-hydroxymethylamino-methane; TRIS-M, Tris-maleate; EDTA, ethylenediamine-tetraacetic acid; Na_2-EDTA, disodium salt of EDTA; AA, ascorbic acid; Cyt, cytochrome; TPNH, reduced triphosphopyridine nucleotide; Q_{O_2}(FW), μl oxygen uptake per hour per gm fresh weight; Q_{O_2}(N), μl oxygen uptake per hour per mg nitrogen; RQ, respiratory quotient.

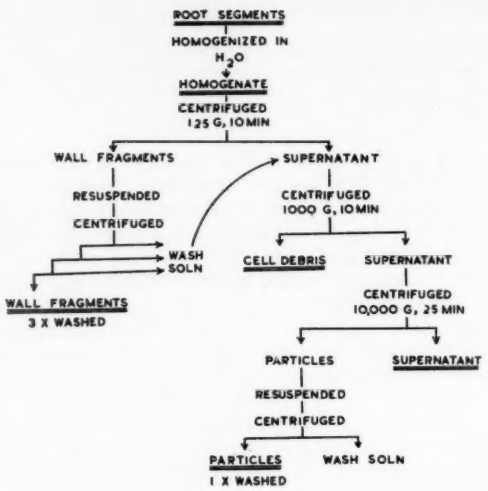

FIG. 1. Procedure for fractionating homogenates of barley root segments. All steps carried out in the cold. Underlined fractions used in enzyme assays.

of segments in each fraction. Nitrogen recovery was also determined in the various fractions.

ENZYME ASSAYS, *Ascorbic acid oxidase:* Activities were determined by manometric methods at 25° C. Of each fraction tested, 0.5 ml was used in 3.0 ml or 3.2 ml final reaction volume. Compositions of the buffer and buffer plus ascorbate were as given for the respiratory measurements. Final pH values of the media after assays were 5.9 to 6.2.

Cytochrome oxidase: Activities were determined by manometric methods at 25° C. One ml of the fractions tested was used in 4.5 ml final reaction volume. Final concentrations of additions were: 2.2×10^{-2} M Na_2-EDTA; 8.8×10^{-2} M phosphate buffer, pH 7.5; 1.8×10^{-1} M sucrose; and 5.7×10^{-5} M cytochrome c when added. Substrate was added from the sidearm as 0.2 ml of 2.23×10^{-2} M hydroquinone in 2.0×10^{-2} M Na_2-EDTA. The amount of hydroquinone added was sufficient to reduce 50 μl of oxygen. The final pH values of the media after assay were 7.0 to 7.2.

For experiments in which mitochondrial yields were determined, the methods described previously for the extraction and assay of succinoxidase and cytochrome oxidase were used (7).

RESULTS AND DISCUSSION

RESPIRATION IN BUFFER: The respiratory rates of roots were not markedly different in the TRIS-maleate or TRIS-EDTA buffers. TRIS-EDTA in which autoxidation of ascorbate was low was preferred. The respiration was measured at about pH 5.8 in order to obtain maximal activities with ascorbate (5).

The effect of age on the root respiration of Cape barley in pH 5.8 TRIS buffers at 25° C (table I) agreed with that reported for Spratt Archer barley roots in pH 7 phosphate buffer at 30° C (12) although the rates of oxygen uptake of the former were about 25 % greater. The decreased rate of oxygen uptake by roots of 10-day-old seedlings was probably related, in part, to a low supply of endogenous substrate since the addition of sucrose or succinate increased the respiratory rate (table I). In the case of roots stored overnight, the difference between respiratory rates in water and in buffer also may be related to low substrate.

The rates of oxygen uptake were of the same magnitude as those of roots of different varieties of barley in water, various buffer solutions, and nutrient solutions (1, 6, 15, 17, 21, 23). One report on oxygen uptake by barley roots (20), about three times as great as other values, differed from these rates.

The respiratory rate of segments from the 10 to 20 mm region was about 30 % less than that of the apical centimeter region but it was about 10 to 20 % less after storage overnight in distilled water at 25° C (table I).

The range of values for the respiratory quotient of root segments (0.73 to 1.00) agreed with other reported values (15, 17, 21).

RESPIRATION IN BUFFER AND ASCORBATE: The respiration of roots in ascorbate was used in testing the barley sensitivity to metal-complexing agents. The apparent ascorbic acid oxidase (the oxygen uptake of roots in ascorbate minus the rate in buffer) was used as an approximation of the potential ascorbic acid oxidase in the intact root segment. The localization

TABLE I

THE OXYGEN UPTAKE OF ROOT SEGMENTS AND THE APPARENT ASCORBIC ACID OXIDASE AT 25° C

AGE (DAYS)	Q_{O_2}(FW)		
	RESPIRATION IN		APPARENT ASCORBIC ACID OXIDASE
	WATER	BUFFER	
3	465	491	799
4	460	469	542
5	378	468	552
5	497	497 *	713
6	494	488 *	....
7	414	469 *	....
9	327	...	....
10	...	231	1190
10	...	289 **	....
10	...	353 †	....
10 + 1 ††	155	291	1090
10 + 1 ††, ‡	142	236	1020
10 + 1 ††, ‡	134	...	....
10 + 1 ††, ‡	333 ‡‡	...	....

* Buffer was 2×10^{-2} M maleic acid adjusted to pH 5.8 with TRIS. In other cases buffer was 5.2×10^{-2} M TRIS + 2.0×10^{-2} M EDTA.
** Buffer plus 0.05 M sucrose.
† Buffer plus 0.04 M succinate.
†† Stored overnight in distilled water at 25° C.
‡ Segments from the 10 to 20 mm root region.
‡‡ In 0.15 M sucrose.

of ascorbic acid oxidase at the cell periphery, the pH optimum of the activity in view of the pK_1 of ascorbic acid (5), and the inhibitions by metal-complexing agents (table III) were consistent with ascorbic acid oxidase being active in the presence of added ascorbate. However, it is possible that cytochrome oxidase, for example, contributed to the oxygen uptake.

Magnitude of the Apparent Ascorbic Acid Oxidase: The activity of the apparent ascorbic acid oxidase ranged from 1.2 to 4.4 times the respiratory rates in buffer. These values were of the same magnitude as those for another variety of barley (5). The Q_{O_2} (FW) values for 5-day roots (table I) were generally lower than those reported for the apparent ascorbic acid oxidase of tobacco pith cells (22). When an allowance was made for endogenous respiration inhibited by ascorbate, the Q_{O_2}(FW) was as great as that of the apparent ascorbic acid oxidase of tobacco pith. In barley the apparent ascorbic acid oxidase was about 25 % of the total extractable ascorbic acid oxidase (table V).

Ascorbate Inhibition of Carbon Dioxide Production: Figure 2 shows that ascorbate not only increased the oxygen uptake but also inhibited the carbon dioxide production by root segments. The ascorbate effects were transitory and disappeared after the induced oxygen uptake equalled the oxidative equivalent of the ascorbate added to the root segments. The rate of carbon dioxide production while ascorbate was being oxidized was significantly different from the rate after the induced oxygen uptake was completed ($\Delta\bar{b} = 0.66 \pm 0.10$ μl CO_2/min × 100 segments, $P < 0.01$). In the presence of ascorbate the carbon dioxide production in five experiments ranged from 71 to 38 % of the rate in buffer alone.

Table II shows an analysis of the ascorbate effects and their modifications by cyanide. Cyanide was used to obtain effects, which could be ascribed to the inhibition of metal enzymes, for comparisons with the

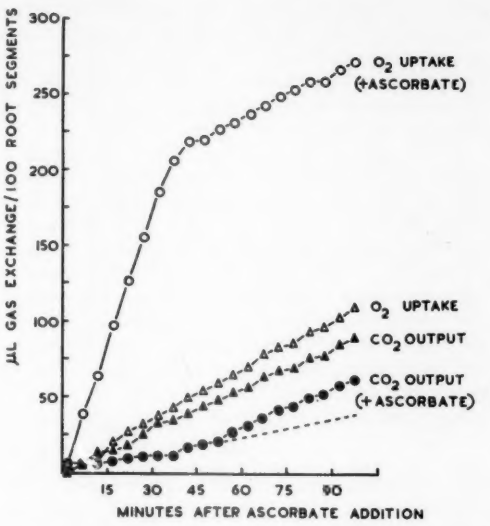

FIG. 2. The effects of ascorbate on the gas exchange of barley root segments in buffer at 25° C. Root segments were from 9-day-old seedlings, stored overnight in distilled water at 25° C.

effects of the so-called copper-complexing agents. Various values for the expected respiratory rates of roots in cyanide plus ascorbate were calculated from the respiratory rates of roots in cyanide and ascorbate separately and were compared with the observed values. In essence, the following interpretations were the basis for making the various assumptions necessary in the calculations. The cyanide effects indicated the presence of at least two terminal oxidase systems, a metal enzyme system and a non-metal enzyme system. The ascorbate effects indicated a change in the relative activities of oxidation and oxidative decarboxylation which presumably was the result of a large

TABLE II

THE EFFECTS OF ASCORBATE AND CYANIDE ON THE GAS EXCHANGE OF ROOTS * AT 25° C

TREATMENT	Q_{O_2}(FW)	Q_{CO_2}(FW)	% OF BUFFER CONTROL		RQ
			Q_{O_2}(FW)	Q_{CO_2}(FW)	
Buffer	421	344	100	100	0.82
Buffer + 10^{-3} M KCN	133	189	32	55	1.42
Buffer + AA	976	133	232	39	0.14
Buffer + KCN + AA	323	253	77	74	0.78
CN inhibited respiration	288	155	68	45	0.54
AA inhibited respiration	257 **	211	61	61	0.82
Apparent ascorbic acid oxidase	555	...	132	...	...
Corrected apparent ascorbic acid oxidase	812 †	...	193	...	...
Calculated KCN + AA + buffer	312 ††	256 ‡	74	74	0.82

* Roots from 4-day seedlings. Ascorbate and cyanide present at the time of respiratory measurement.
** Assuming the RQ of the respiration inhibited by AA is 0.82.
† Correcting for the endogenous respiration inhibited by AA.
†† Assuming cyanide affects the respiration of roots in ascorbate as it does that of roots in buffer alone.
‡ See text for method of calculation.

supply of hydrogen atoms and electrons being made available. Therefore, the addition of both cyanide and ascorbate to roots could affect, by inhibition and mass action, the pathway of oxidation and the relative amounts of oxidation and oxidative decarboxylation.

Since ascorbate inhibited CO_2 production, it probably also inhibited the endogenous oxygen uptake although its action was masked by the oxidation of ascorbate itself. Thus, the calculated value of the apparent ascorbic acid oxidase (oxygen uptake in ascorbate minus the rate in buffer alone) was too small. The endogenous respiration remaining when ascorbate was added to the roots was calculated by assuming the RQ of the respiration inhibited by ascorbate was the same as the endogenous respiration. The corrected value was thus:

$$421\ Q_{O_2}(FW) - 211\ Q_{CO_2}(FW)/0.82\ Q_{CO_2}(FW) \times Q_{O_2}(FW)^{-1} = 161\ Q_{O_2}(FW).$$

The corrected ascorbic acid oxidase value, 812 $Q_{O_2}(FW)$, was 46 % greater than the uncorrected value.

Cyanide incompletely inhibited oxygen uptake and carbon dioxide production (table II). Thus, it cannot be assumed that cyanide completely reversed the ascorbate inhibition of CO_2 production. If the latter did occur, the expected CO_2 production in cyanide plus ascorbate would be the same as the CO_2 production of roots in cyanide alone, 189 $Q_{CO_2}(FW)$. However, since the oxygen uptake of roots in cyanide plus ascorbate was increased over that of roots in cyanide alone, 189 $Q_{CO_2}(FW)$ was a minimal value which was supplemented by CO_2 production associated with the increased oxygen uptake. The increased oxygen uptake probably resulted from both the cyanide resistant fraction of the oxidation of ascorbate itself and, owing to the cyanide reversal of the ascorbate effects, the cyanide resistant fraction of the endogenous respiration which was not inhibited by ascorbate.

For CO_2 production of roots in cyanide plus ascorbate, it is assumed that oxidative decarboxylation of ascorbate itself was nil. In earlier experiments from another laboratory similar to these for barley (22), it was found that ascorbate added to tobacco pith cells was quantitatively recovered as dehydroascorbate. Calculation of the expected CO_2 production depends upon the estimation of the cyanide resistant fraction of the endogenous respiration which was inhibited by ascorbate, i.e., the amount of endogenous respiration which occurred in cyanide because cyanide reversed the effects of ascorbate. Several methods of calculations are possible. One is presented to illustrate that the observed value of CO_2 production was, in fact, not unexpected. Assuming that the RQ of the respiration inhibited by ascorbate was 1.0 (e.g., oxidation in the Krebs cycle) and therefore the true cyanide resistant fraction of respiration was shown by the cyanide resistant fraction of oxygen uptake, the expected CO_2 production of roots in cyanide plus ascorbate is:

$$189\ Q_{CO_2}(FW) + 0.32 \times 211\ Q_{CO_2}(FW) = 256\ Q_{CO_2}(FW).$$

TABLE III

THE EFFECTS OF METAL-COMPLEXING AGENTS ON THE OXYGEN UPTAKE OF ROOT SEGMENTS AND THE APPARENT ASCORBIC ACID OXIDASE AT 25° C

AGE (DAYS)	BUFFER *	PRETREATMENT **	TREATMENT	% INHIBITION		
				RESPIRATION IN		APPARENT ASCORBIC ACID OXIDASE
				BUFFER	AA + BUFFER	
5	TRIS-EDTA	10^{-3} M DDC	.....	33	76	92
5†	TRIS-M	.....	5×10^{-4} M PTU	..	15	$\leqslant$ 23
5	TRIS-M	.....	1.5×10^{-3} M PTU	..	8	$\leqslant$ 30
5	TRIS-EDTA	.....	1.5×10^{-3} M PTU	4	19	31
5 + 1	PO_4	10^{-2} M PTU	.....	60	23	+ 7
6	TRIS-M	.....	10^{-3} M PTU	+ 3	..	..
7	TRIS-M	.....	5×10^{-4} M PTU	4	..	..
9 + 1	TRIS-EDTA	10^{-3} M DDC	.....	46	90	97
10	TRIS-EDTA	10^{-3} M DDC	.....	34	88	94
10 + 1	TRIS-M	10^{-4} M 8-OH-Q	.....	14	12	12
10 + 1†	....	10^{-4} M 8-OH-Q	.....	+ 17 ††	..	..
"	....	"	0.15 M sucrose	56 ††, ‡	..	..
10 + 1†	TRIS-EDTA	10^{-3} M 8-OH-Q	.....	73	57	53
"	"	"	1.5×10^{-3} M PTU	..	82	$\leqslant$ 85
"	"	"	10^{-3} M KCN	..	95	$\leqslant$ 100

* Phosphate buffer: 0.17×10^{-1} M, pH 6.0. Other buffers as described previously.

** Diethyldithiocarbamate pretreatment of 45 min soak in freshly prepared DDC in buffer, pH 5.8. Phenylthiourea and 8-hydroxyquinoline pretreatment of 17 to 20 hrs soak at 25° C.

† Segments from the 10 to 20 mm root region.

†† No buffer present.

‡ Inhibition of the net sucrose stimulation, the latter being 15 %.

Since glycolysis and the hexose monophosphate shunt may possibly contribute CO_2 to a greater extent in cyanide treatments compared with their possible contributions to the aerobic endogenous root respiration, the observed value for the cyanide resistant CO_2 production was not used.

It is apparent that different calculated values can be obtained arbitrarily. Therefore, no special significance is placed upon them except to show that combined cyanide plus ascorbate effects could be explained if appropriate considerations were made. Since the CO_2 relationships were beyond the scope of this investigation, only the effects of inhibitors on oxygen uptake were further studied. The values for inhibitions presented in table III were based upon comparisons of oxygen uptakes with or without inhibitors or inhibitor pretreatment in the cases of respiration of the roots in buffer or buffer plus ascorbate. For the apparent ascorbic acid oxidase, the inhibition was calculated from the comparisons of the apparent ascorbic acid oxidase (oxygen uptake in ascorbate minus the rate in buffer alone) with the oxygen uptake in ascorbate plus inhibitor minus the respiratory rate of the roots in the inhibitor.

Explanation of the Ascorbate Effects: The effects of ascorbate can be explained in at least two ways. Ascorbate may replace some portion of the endogenous substrate oxidized through the normal pathways. Alternatively, ascorbate may inhibit a portion of respiration until the ascorbate is oxidatively converted into a non-inhibitory form by a system which normally may or may not contribute to oxygen uptake.

If ascorbate replaced endogenous substrate the production of carbon dioxide would be expected to decrease if known metal oxidases were involved since the initial dehydrogenations of ascorbate, for example by cytochrome c or ascorbic acid oxidase, do not involve decarboxylations such as are associated with the dehydrogenations of endogenous substrate. Increased oxygen uptake, as in the alternative explanation, can be attributed to an increased supply of reduced substrate. For purposes of discussion, metal oxidase respiration is identified with the respiratory fraction which is inhibited by cyanide or by ascorbate and with the oxidation of ascorbate itself. The metal oxidase respiration estimated from 10^{-3} M cyanide inhibition and the maximal ascorbate inhibition was 65 to 70 % of the total respiration. Of the metal oxidase respiration, it can be estimated from the effects of 10^{-3} M diethyldithiocarbamate (table III) that not more than half was mediated by copper enzymes. From the maximal inhibitions by 10^{-2} M phenylthiourea and 10^{-3} M 8-hydroxyquinoline (table III), it can be inferred that almost the entire fraction was mediated by copper enzymes if their specificity towards copper were assumed. However, these different degrees of inhibition by diethyldithiocarbamate, phenylthiourea, and 8-hydroxyquinoline are evidence indicating their non-specificity for copper enzymes.

Ascorbate was reported to inhibit a non-particulate TPNH-oxidase system from wheat germ (8), presumably by reduction of a cofactor active only in the oxidized form. A fraction of the respiration of barley roots may pass through a system like the wheat flavin TPNH-oxidase. It may be inhibited by ascorbate, in the manner suggested for the wheat germ system, until the ascorbate itself is oxidized, possibly by ascorbic acid oxidase. Since the soluble TPNH-oxidase from wheat germ was inhibited by cyanide and diethyldithiocarbamate (8), which also inhibit ascorbic acid oxidase, additional difficulties in the estimation of the quantitative role of ascorbic acid oxidase with inhibitors become evident.

The Effects of Metal-complexing Agents, *Diethyldithiocarbamate:* Table III shows that pretreatment with diethyldithiocarbamate inhibited the apparent ascorbic acid oxidase by about 95 %, a much greater effect than on respiration. Although more inhibitory to the apparent ascorbic acid oxidase than other copper complexing agents, diethyldithiocarbamate inhibited respiration only half as effectively as phenylthiourea or 8-hydroxyquinoline under conditions facilitating maximal inhibitions. Respiration of roots from both 5- and 10-day seedlings were usually inhibited by about 35 to 45 %. These respiratory inhibitions were lower than those previously reported, which ranged from 49 to 59 % for 5-day-old roots, and 34 to 58 % for 10-day-old roots (12).

Diethyldithiocarbamate has been used as a specific inhibitor of ascorbic acid oxidase in vivo (12, 13). However, it has been stressed that diethyldithiocarbamate is not specific for copper, that it can inhibit succinic dehydrogenase (4), and may complex with copper enzymes in respiratory systems terminating with cytochrome oxidase (6).

Evidence from a study of dithiocarbamyl compounds indicates that many of their effects can be attributed to inhibitions of thiol-activated enzymes, including succinic dehydrogenase (3). Some evidence was presented to show that the dithiocarbamyls also form complexes between copper and proteins whose stabilities are greater than those of the complexes with other metals. Such protein-copper-dithiocarbamyl complexes can account for the marked irreversible in-

Table IV

Effects of Diethyldithiocarbamate Pretreatment on the Respiration of Root Segments * Treated with Malonate and Succinate

Treatment	Q_{O_2}(FW)		% of control	
	Buffer	DDC **	Buffer	DDC
Sucrose 0.05 M ***	289	257	100	89
Malonate 2×10^{-2} M	237	243	82	84
Malonate + succinate	308	250	107	87
Succinate 4×10^{-2} M	353	275	122	95

* Roots from 10-day-old seedlings.
** Diethyldithiocarbamate pretreatment of 45 min soak in freshly prepared DDC in buffer, pH 5.8.
*** All treatments contain sucrose and TRIS-EDTA buffer, pH 5.8.

hibitions of ascorbic acid oxidase by diethyldithiocarbamate. Ascorbic acid oxidase in barley roots was markedly inhibited by diethyldithiocarbamate since the activity of the apparent ascorbic acid oxidase was decreased (table III) and the activity of ascorbic acid oxidase isolated from treated roots was diminished (table VI). However, the inhibition of respiration probably cannot be attributed solely to inhibition of ascorbic acid oxidase. Table IV presents evidence that some effects of diethyldithiocarbamate can be attributed to the inhibition of succinic dehydrogenase in vivo. Malonate inhibition of respiration was partially reversed by succinate, which is evidence for the participation of succinic dehydrogenase in respiration. Diethyldithiocarbamate diminished the apparent malonate inhibition of respiration. In addition, the increase in respiration induced by succinate was diminished as was also the succinate reversal of the malonate inhibition.

Phenylthiourea and 8-Hydroxyquinoline: Root segments were soaked for 17 to 20 hours in phenylthiourea and 8-hydroxyquinoline prior to respiratory measurements. The prolonged pretreatments were used to provide sufficient time for the agents to exert their maximal effects.

Phenylthiourea inhibition of the apparent ascorbic acid oxidase without inhibition of respiration (table III) was consistent with the action of a substance not rapidly entering cells but still acting upon a surface localized enzyme. On the other hand, pretreatment with high concentrations of 10^{-2} M phenylthiourea and 10^{-3} M 8-hydroxyquinoline could inhibit respiration to the same extent as cyanide but without comparable inhibitions of the apparent ascorbic acid oxidase. Rinsing of the pretreated roots may have partially reversed the effects on the surface localized ascorbic acid oxidase without removing the agents which may have entered the cytoplasm.

The respiration of roots from 10-day-old seedlings was only slightly inhibited or was even increased by 10^{-4} M 8-hydroxyquinoline. When the respiration was increased by additions of sucrose, the inhibitory effects were markedly shown (table III). It appears that in barley roots there is a portion of respiration relatively insensitive to metal-complexing agents and which persists in spite of low substrate supply. Any increase in respiration induced above this basal rate, whether by salt (21) or substrate, is qualitatively different.

Cyanide: In most experiments the respiration was inhibited by 10^{-3} M cyanide by about 65 to 70 % (table II). These inhibitions equalled some previously reported values for barley roots (12, 23) and were slightly greater than others (16, 21). In combination with 8-hydroxyquinoline the oxygen uptake of roots in ascorbate was inhibited by 95 %. This combination, as also with phenylthiourea instead of cyanide, resulted in inhibitions greater than expected on the bases of either maximal effects of any one of the agents or the sum of the individual effects of comparable treatments.

Isolation of Ascorbic Acid Oxidase and Cytochrome Oxidase: It is desirable to confirm the effects of respiratory inhibitors by determining their effects on the respiratory enzymes isolated from the treated tissue. This is feasible only if the inhibitory effects are irreversible, as in the case of diethyldithiocarbamate (13). If the activity of the extracted oxidases from treated roots is insufficient to account for the respiration of the untreated tissue, the results are consistent with the effects of the inhibitors on respiration. On the other hand, if the activity is sufficient to account for the respiration of the untreated tissue, it is doubtful whether the effects of the inhibitors on respiration can be solely attributed to their effects on the oxidases examined.

Ascorbic Acid Oxidase: The major portion of the ascorbic acid oxidase was associated with the cell wall fragments (tables V and VI) as previously reported for another variety of barley (5). The particulate mitochondrial fraction contained only about 1 % and the soluble or supernatant fraction about 3 % of the total ascorbic acid oxidase (table V). The soluble ascorbic acid oxidase, allowing for the changes in re-

Table V

The Recovery of Ascorbic Acid Oxidase and Nitrogen in Fractions of Root Homogenates *

Fraction	Assay	Equivalent Q_{O_2}(FW) from 1 gm roots	Activity recovery, %	Nitrogen recovery, %	Q_{O_2}(N)
Root segments	Respiration	333	...	...	104
"	Corrected apparent ascorbic acid oxidase	1530	...	...	481 (2050 **)
Homogenate	Ascorbic oxidase	5730	100	100	2220
Wall fragments	"	3650	63.9	24	5790
Cell debris	"	90	1.6	3	1230
Superspeed supernatant	"	171	3.0	32	205
Particles	"	78	1.3	23	123
Total recovery in fractions	"	3990	69.8	84 ***	....

* Root segments from 9-day-old seedlings, stored overnight at 25° C in aerated distilled water.
** Computed on the basis of nitrogen in the wall fraction.
*** Including other fractions.

TABLE VI

THE RECOVERY OF ASCORBIC ACID OXIDASE AND CYTOCHROME OXIDASE FROM ROOT SEGMENTS

FRACTION	ROOT PRETREATMENT	ASSAY	EQUIVALENT Q_{O_2}(FW) FROM 1 GM ROOTS		% OF RESPIRATION
			− CYT C	+ CYT C	
Root segments *	Buffer	Respiration	491	...	100
Homogenate **	"	Ascorbic oxidase	3830	...	760
Wall fragments **	"	"	4650	...	946
Homogenate **	"	Cytochrome oxidase	0	719	146
Wall fragments **	"	"	0	141	29
Particles **	"	"	0	338	69
Root segments ***	Buffer	Respiration	125	...	100
"	10^{-3} M DDC	"	83	...	66
"	Buffer	Apparent ascorbic acid oxidase	1160	...	928
"	10^{-3} M DDC	"	66	...	53
Homogenate **	10^{-3} M DDC	Ascorbic acid oxidase	189	...	151
"	"	Cytochrome oxidase	28	161	106

* From separate experiments with roots from 3-day-old seedlings.
** Fractions from homogenates of entire root systems of 3-day-old-seedlings.
*** Root segments from 10-day-old seedlings.

covery under conditions tending to elute it from the cell wall (5), could just account for the respiration of root segments while the total ascorbic acid oxidase was more than adequate (tables V and VI). The total activity of ascorbic acid oxidase isolated from roots pretreated with diethyldithiocarbamate could account for the respiration of even untreated root segments (table VI). However, the ascorbic acid oxidase in the non-cell wall fractions may have been sufficiently inhibited to reduce the respiration if it were mediated by that portion of the ascorbic acid oxidase.

Considerations of only the soluble portion of the ascorbic acid oxidase for the quantitative assessment of respiration (19) probably neglects the major source of the enzyme, which is associated with the cell wall in many plant species (5, 18, 22). An attempt by other investigators (19) was made to show that a soluble ascorbic acid oxidase, linked to a soluble TPN reductase by various steps, did account for the respiration of pea cotyledons in vivo. Its role in detached embryos was questionable (19). It is possible that the major source of ascorbic acid oxidase from pea seedlings was not assessed since the fraction of homogenates containing the cell wall fragments was not studied. This point is critical since the activity of the system was limited by ascorbic acid oxidase. The correspondence of the respiratory activity of extracts with the respiration of the intact cotyledons may well have been fortuitous, as was recognized by the investigators (19).

Cytochrome Oxidase: Cytochrome oxidase was largely in the particulate fraction but a relatively large proportion still was detected with the cell wall fraction in this experiment (table VI). Presumably this indicated that quantities of mitochondria were trapped with the cell wall fragments. The activity of cytochrome oxidase from apical segments was sufficient to account for respiratory rates. Particulate succinoxidase and cytochrome oxidase isolated from entire root systems (table VII) by a different method, however, could not account for the respiratory rates. The differences of importance consisted mainly in pH and composition of the isolation media since the stepwise procedure was identical. A previous investigation (2) has reported the inadequacy of cytochrome oxidase activity to account for barley root respiration. The positive results here with cytochrome oxidase suggest that other failures were due largely to inappropriate methods.

The unfractionated homogenate clearly showed cytochrome oxidase activity (table VI). For other varieties of barley, cytochrome oxidase could not be

TABLE VII

RECOVERY OF PARTICULATE SUCCINOXIDASE AND CYTOCHROME OXIDASE FROM ROOTS

AGE (DAYS)	NO. EXPTS	PARTICLES % ROOT WT *	EQUIVALENT Q_{O_2}(FW) FROM 1 GM ROOTS **	
			SUCCIN-OXIDASE	CYT. OXIDASE
2	20	3.1 ± 0.16 [a]	88	246
3	5	3.5 ± 1.25	98	278
4	1	2.0	56	160
5	4	2.9 ± 0.40 [b]	82	232
6	1	2.0	56	160
10	5	2.0 ± 0.47 [a, b]	56	160

* Standard errors corrected for number of experiments.
** Activities calculated from the particle recovery values and the activities assayed as described previously (7) asuming 50 % recovery of activity with the particles (cf. table VI).
[a, b] Pairs of figures with the same symbols significantly different from each other, $P < 0.05$.

unequivocally shown in unfractionated homogenates of roots (2, 5) although it was demonstrated with the particles (5). Thus, varietal differences may account for reports of the lack of cytochrome oxidase in unfractionated homogenates, i.e., homogenates only cleared by centrifugation at $500 \times g$ (12); but it seems unlikely that cytochrome oxidase is absent from the roots. The choice of substrate may also be important for unfractionated homogenates owing to the presence of other oxidases.

CONCLUSIONS: The quantitative assessment of the role of oxidases in barley roots from the degree of inhibition, especially by diethyldithiocarbamate, can scarcely be simple. This is emphasized by the isolation of ascorbic acid oxidase from roots treated with diethyldithiocarbamate in sufficient amounts to account for the respiration of untreated roots. In addition, under conditions facilitating maximal effects, inhibitions of respiration to different degrees by different complexing agents often considered specific for copper (11) suggests the non-specificity of the agents for copper enzymes in vivo. That thiol enzymes, such as succinic dehydrogenase, were possibly inhibited further suggests the non-specificity of diethyldithiocarbamate for metal enzymes. These results indicate that the assessment of the quantitative role of ascorbic acid oxidase by inhibitors is not completely satisfactory. The possible role of cytochrome oxidase cannot be neglected since it is present in ample quantity to account for the cyanide sensitive portion of respiration of barley roots. Lack of photo-reversible inhibition by low partial pressures of carbon monoxide in low oxygen tensions cannot be considered to demonstrate the lack of cytochrome oxidase activity unequivocally. Experiments with high positive pressures of carbon monoxide, as with potato tuber (25), are desirable in order to establish whether cytochrome oxidase is functional in roots of older barley plants.

SUMMARY

The respiratory rates in water, buffer, and buffer plus ascorbate, of root segments from barley seedlings of different ages are reported. Ascorbate not only increased the oxygen uptake but also inhibited the carbon dioxide production. Both effects were transitory and disappeared after the increased rate of oxygen uptake accounted for the oxidative equivalent of the ascorbate added. The respiration inhibited by ascorbate, as measured by lowered carbon dioxide production, was about equal in magnitude to the cyanide inhibited portion of oxygen uptake.

Maximal respiratory inhibitions by 8-hydroxyquinoline and phenylthiourea were about the same as the degree of cyanide inhibition. Sodium diethyldithiocarbamate inhibited respiration about one-half as effectively as the above reagents. Malonate inhibition of roots pretreated with diethyldithiocarbamate was low or absent in comparison with that of untreated roots.

The apparent ascorbic acid oxidase, i.e., oxygen uptake in ascorbate minus the rate in buffer alone, was used as an approximate measure of ascorbic acid oxidase activity in the intact root segment. It amounted to about one-fourth the ascorbic acid oxidase activity which could be found in homogenates of the root segments. Diethyldithiocarbamate inhibited the apparent ascorbic acid oxidase to about 95% while 8-hydroxyquinoline and phenylthiourea were less inhibitory.

Both cytochrome oxidase and ascorbic acid oxidase in amounts sufficient to account for the respiration of untreated root segments could be isolated from segments treated with diethyldithiocarbamate.

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THE INDOLE-3-ACETIC ACID OXIDASE OF LUPINUS ALBUS L.[1]

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Although there have been numerous studies on indole-3-acetic acid (IAA) oxidase since its existence was suggested by Thimann (17) and demonstrated by Larson (9), the reports on the composition, mode of action, and reaction products are conflicting and incomplete. Tang and Bonner (18) using etiolated pea epicotyls as the source of the enzyme, concluded that the oxidase contained an iron porphyrin group. Wagenknecht and Burris (20) suggested that the IAA oxidase from bean roots is a copper protein; they demonstrated stimulation by Mn^{++} and considered indole-3-aldehyde as the main reaction product. Gortner and Kent (5) found an IAA oxidase in pineapple meristem which could be differentiated from others by its low pH optimum of activity and by its response to certain phenolic inhibitors. A significant advance was achieved by Goldacre et al (3) who observed stimulation of IAA oxidase from etiolated pea epicotyls by catalytic amounts of 2,4-dichlorophenol. Galston (1) suggested that the enzyme is a light-activated flavoprotein-linked peroxidase, and the peroxidase concept has found further support in the experiments of Kenten (6) and Waygood (21). Kenten observed that highly purified horseradish peroxidase in the presence of phenolic activators and Mn^{++} oxidises IAA.

Much of the inconsistency concerning the nature of the enzyme or enzymes involved in the oxidation of IAA may stem from the disparity of the sources investigated in the past, which has been further complicated by the presence of activators and inhibitors. Furthermore, the enzymatic decomposition of IAA results in at least four major products, none of which has been adequately characterized.

After a preliminary survey, *Lupinus albus* L. was selected as the source material because it appears that the enzyme from this plant is more stable, and can be obtained with ease in satisfactory yields from both etiolated and non-etiolated tissue.

A sequence of relatively simple fractionation steps which permit a thousand-fold concentration is presented here. The properties of this preparation are reported.

MATERIALS AND METHODS

Besides *Lupinus albus* L., the following plants were examined as potential sources of the enzyme: roots, stems, and leaves of *Phaseolus vulgaris* L. var. Red Kidney, roots of 3-day-old etiolated seedlings of *Avena sativa* L. var. Victory, primary leaves and apical buds of 7-day-old seedlings of *Phaseolus aureus* Roxbg., cocklebur leaves (*Xanthium* sp.), potato tubers (*Solanum tuberosum* var.), tobacco pith (*Nicotiana Tabacum* var. Lucky Strike Suckerless) and etiolated epicotyls of *Pisum sativum* var. Early Alaska.

Etiolated lupine plants were grown on water culture in the dark. The seeds were soaked for approximately 3 hours in tap water, and germinated in large Petri dishes in the dark at 25° C. After 5 days the seedlings were placed on a slotted lucite sheet with the roots extending into a tray of water. If photoeffects were to be studied, this planting operation was carried out under a dim red or green safelight (1) whichever was to be used subsequently. Hypocotyl elongation (growth) was permitted until the 10th to

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12th day in darkness at 85 % relative humidity and 25° C.

The non-etiolated (green) plants were grown either in a greenhouse on soil, or in a plant growth room on ¼-inch quartz gravel in beds which were irrigated with nutrient solution. The room was kept at 25° C, between 60 and 70 % relative humidity; the photoperiod was 12 hours, and the illumination was about 3000 ft-c.

The results of the present study indicate that the same or an enzyme of similar function is obtained from both the hypocotyls and epicotyls of the etiolated seedlings and from the aerial parts of non-etiolated plants. Green tissue was the preferred source of the enzyme, because the concentration of enzyme was higher than that in etiolated tissue.

PURIFICATION OF THE ENZYME: Any convenient amount of tissue (10 to 1000 gm; fresh or previously frozen) was ground in a chilled porcelain mortar with the aid of a small amount of quartz sand; one volume of water was added to non-etiolated preparations during grinding. The extract was expressed through several layers of cheesecloth, and centrifuged at 20,000 × g for 10 minutes; this preparation is stable towards freezing. The extract was dialyzed for 18 to 36 hours, first against running tap water and finally against distilled water to remove the naturally occurring inhibitors. The precipitate which formed in the crude preparations on dialysis, standing in the cold room, or thawing could be centrifuged off with very little loss of activity.

After dialysis, the enzyme, at not less than 3 % protein concentration, was adjusted to 80 or 100 % saturation with solid ammonium sulfate, with mechanical stirring at low temperature, and centrifuged. The precipitate was resuspended in a minimum volume of water and both it and the supernatant were freed of ammonium sulfate by dialysis. The dialyzed supernatant was concentrated at low temperature, and both fractions were frozen or lyophilized. Although a large fraction of the oxidase is soluble in high concentrations of ammonium sulfate, the distribution of activity varies both with the concentration of the salt and with tissue used (table I).

Further purification of the enzyme was achieved by electrophoresis (fig 1), using the technique and apparatus described by Kunkel and Slater (8). The migration of the protein fractions was usually carried out in a starch bed saturated with 0.025 M phosphate buffer (pH 7.0). The enzyme was introduced as a slurry of starch in buffer into a trench in the bed, placed at about 3/5 of the total distance from the negative electrode; 10 to 50 mg of protein was easily accommodated in a starch bed of 5 cm^2 cross section. The electrophoresis was carried out at 300 volts and 12 to 15 milliamperes for 18 hours at 5° C. At the end of the experiment the block was cut into 1-cm segments, each of which was extracted with 5 ml of water in a centrifuge tube; the supernatant fractions were used for enzyme and protein determinations. Active fractions were kept in the frozen state; little decrease in activity was observed after one month. The efficiency of the purification procedure is shown in table I.

TABLE I

PURIFICATION OF INDOLEACETIC ACID OXIDASE AND PURPUROGALLIN PEROXIDASE FROM LUPINUS ALBUS L.

FRACTION EXAMINED	ETIOLATED TISSUE		NON-ETIOLATED TISSUE					
	EPICOTYLS AND HYPOCOTYLS		LEAVES		STEMS		HYPOCOTYLS	
	$Q_{O_2}(N)$	PZ′	$Q_{O_2}(N)$	PZ′	$Q_{O_2}(N)$	PZ′	$Q_{O_2}(N)$	PZ′
Crude centrifuged extract	19	0.6	< 1 *	1.5	1 *	2.4	138	1.1
Ether extracted crude solution	....	...	313	5.9	....	....	....	...
Dialyzed and recentrifuged	416	1.9	200	3.4	700	9.5	1440	9.5
			$(NH_4)_2SO_4$-treated fractions					
80 % sat., precipitate	134	0.6	237	1.6	67	1.2	285	0.4
80 % sat., supernatant	2400	8.2	5,040	50.0	1,700	20.0	3500	8.2
80 % sat., supernatant, after electrophoresis **	....	...	130,000 190,000	600.0 1100.0	....	....	....	...
100 % sat., precipitate	283	2.6	230	3.4	2,150	17.0	1360	5.7
100 % sat., precipitate, after electrophoresis	....	...	26,000	135.0	....	....	....	...
100 % sat., supernatant	1	4.3	1	1.1	3,600	55.0	6000	20.0
100 % sat., supernatant, after electrophoresis	....	...	....	....	87,000	450.0	....	...

* Inhibited by natural inhibitors. The presence of these inhibitors in the crude extract makes it impossible to calculate enzyme yields. IAA oxidase activity is indicated by the $Q_{O_2}(N)$ calculated on the basis of the best 10-min value (usually the second 10-min interval) for oxygen uptake for the mg of enzyme protein used. PZ′ (a modified purpurogallin number) is defined under Methods.

** Upper values, electrophoresis in orthophosphate buffer at pH 7; lower value, in acetate buffer at pH 5.5.

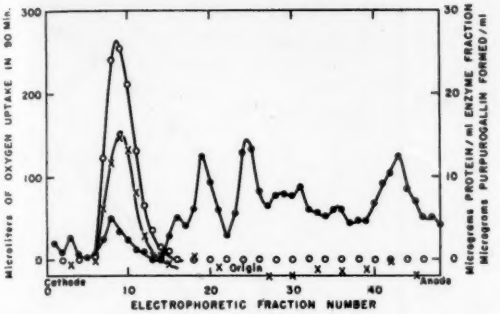
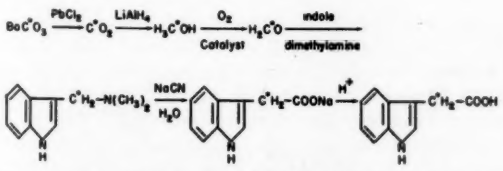


FIG. 1. Oxidase, peroxidase and protein distribution on electrophoresis: ●, μgm protein per ml eluate from the electrophoretic fraction; ○, μgm purpurogallin formed per ml reaction mixture (0.03 ml enzyme); ×, μl oxygen uptake in 90 minutes (0.5 ml enzyme). Fractionation by starch block electrophoresis in 0.025 M pH 5.5 sodium acetate buffer.

DETERMINATION OF IAA OXIDASE: The activity of the enzyme was measured by the Warburg manometric method (19). Each flask contained 1.0 ml of 0.4 M pH 6.3 buffer, usually orthophosphate, 1 ml of enzyme solution, 30 micromoles of IAA as the sodium salt, 3 micromoles of Mn^{++}, and 0.3 to 3 micromoles of 2,4-dichlorophenol (3); water was added to give a volume of 3 ml, and finally, 0.1 ml M KOH was placed with a wick in the center well. Air was the gas phase. The operating pH of this system was between 6.3 and 6.5.

For the determination of IAA oxidase activity, the Salkowski assay (4) for residual IAA was not used because three of the reaction products give positive tests. Platt and Thimann (11) have recently documented other difficulties which interfere with the specificity of this assay. However, a solution of 0.5 % $FeCl_3$ in 2 N $HClO_4$ was found to be very useful as a chromatographic spray for IAA and some of the oxidation products.

Carboxyl-C^{14} IAA and IAA-2-C^{14} were synthesized following modifications of the procedure of Snyder and Pilgrim (14, 16). The dimethylamine group of gramine was replaced by C^{14}-NaCN to give the carboxyl-labeled IAA. The reaction sequence for the IAA-2-C^{14} (16) synthesis was:



Because of radiodecomposition both labeled substrates were purified chromatographically.

Protein values were estimated either by Nesslerization (19), or by the modified Folin method of Lowry (10).

DETERMINATION OF PEROXIDASE: Because earlier work (1, 6) had suggested a peroxidase action of IAA oxidase, it was necessary to test the lupine enzyme for this activity. This was done colorimetrically by a modified Willstätter and Stoll procedure (15, 22). The enzyme preparation was mixed with 1.25 ml of 0.4 M phosphate buffer pH 6.3, 2.5 ml of freshly prepared 0.5 % pyrogallol solution, and water to 4.5 ml, and the mixture was allowed to equilibrate at 20° C. One-half ml of 0.06 % H_2O_2 was added at time zero with mixing and the reaction was stopped after ten minutes by the addition of 0.2 ml of 2 N H_2SO_4.

The standard purpurogallin curve was prepared by dissolving purpurogallin in acetone and reading appropriate aliquots in the Klett-Summerson photoelectric colorimeter with the No. 42 filter (420 mμ). Linearity is obtained up to saturation with purpurogallin.

Willstätter and Stoll (22) defined the purpurogallin number as

$$PZ = \frac{\text{mg purpurogallin in 500 ml}}{\text{volume of enzyme} \times \text{dry weight}}$$

Although a number of simplifications of this test have been reported (Sumner (15) and others), the more direct determination of the purpurogallin number given above was developed, because it facilitates greatly the assay of large numbers of samples.

To make the values comparable to the older PZ values, the calculations were adjusted to the 500-ml level with the substitution of mg of protein for dry weight. However, there remains the difference in procedure, and to avoid confusion with the values obtained by Willstätter's and Stoll's method, the present results are expressed as PZ′ numbers. Table II shows representative data.

Horseradish peroxidase and twice-crystallized catalase were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey; and crude glucose oxidase was obtained from the Sigma Chemical Co., St. Louis, Missouri.

TABLE II

FORMATION OF PURPUROGALLIN BY LUPINE PEROXIDASE

SOURCE AND PURIFICATION OF ENZYME	PROTEIN USED	PURPUROGALLIN FORMED	PZ′
	μgm	*μgm/ml*	
Leaves; precipitated with	0.13	2.3	89
$(NH_4)_2SO_4$ (100 % sat.);	0.39	7.5	96
purified by electrophoresis	0.78	14	90
	1.3	23	89
Stems; soluble in sat.	0.13	7.1	270
$(NH_4)_2SO_4$ solution;	0.39	20	260
purified by electrophoresis	0.78	37	240
	1.3	55	210
Extract from leaves dialyzed	40	5.5	1
and centrifuged	120	23	0.96
	240	40	0.83
	2000	136	0.34

Results

The pH responses of a crude extract and of an electrophoretically purified preparation are shown in figure 2. The shift in pH optimum after purification reflects the removal of the natural inhibitors and replacement of the natural activators with 2,4-dichlorophenol. No difference in the pH activity relationship was found between enzymes from hypocotyls and from other aerial parts; likewise, no difference was found between plant material from 4- and 6-week-old plants. Exhaustively dialyzed preparations usually have an optimum of pH 6.3 instead of the pH 6.5 of the electrophoretically purified preparation.

The heat-stable natural activator can be extracted with ether from the crude enzyme solution or from the concentrated dialyzates. Its phenolic nature is suggested by a positive $FeCl_3$ test and by its infrared absorption spectrum. Varying amounts of inhibiting substances are often present in the ether extracts of the activator as shown by concentration studies. Therefore, its identification has been postponed and 2,4-dichlorophenol (3) has been used as a substitute (see fig 3 and table III).

Crude enzyme preparations show a lag in activity, which was abolished by dialysis (fig 4). The effect of the natural inhibitor can be simulated by low concentrations of some phenols such as guaiacol and pyrogallol which induce a lag in oxygen uptake similar to that observed with some crude enzyme preparations from green tissues; the same effect was observed by Kenten (6) for pyrogallol with horseradish peroxidase. Other phenols, hydroquinone, 3,4-dihydro-

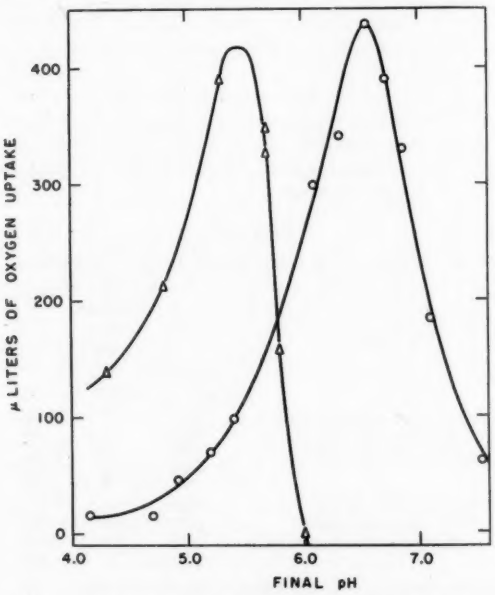


Fig. 2. The effect of pH on oxygen uptake after 90 min: △, crude enzyme extract, ○, electrophoretically purified enzyme—both from non-etiolated tissue.

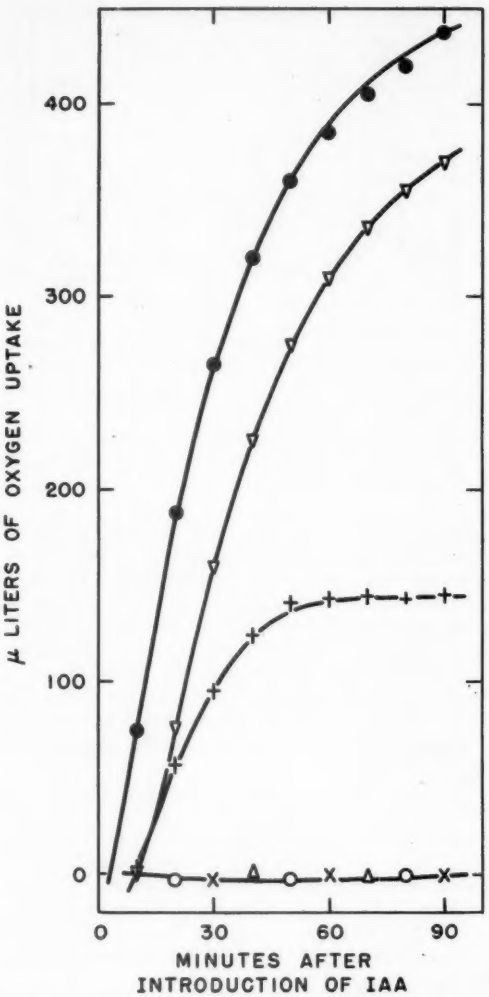


Fig. 3. The effect of Mn^{++}, 2,4-dichlorophenol, and natural activator on IAA oxidase activity: ○, boiled enzyme + IAA; ×, enzyme + IAA; △, enzyme + IAA + Mn^{++}; +, enzyme + IAA + 2,4-dichlorophenol; ●, enzyme + IAA + Mn^{++} + 2,4-dichlorophenol; ▽, enzyme + IAA + Mn^{++} + ether extract of dialysate.

coumarin, eugenol, catechol, 3,4-dihydroxyphenylalanine, protocatechuic acid, and caffeic acid, were tested because it was thought that they might be identical to the natural inhibitor or exert similar effects. All were totally inhibitory at a concentration of 10^{-4} M.

Besides manganese ions, iron, cobalt, copper, magnesium, and calcium ions were examined for their effect on the system. None was found to be capable of replacing manganese (fig 5).

In the absence of phenolic activator, Mn^{++} was without effect on the oxygen uptake, but in the presence of 3 micromoles of 2,4-dichlorophenol 0.3 to 30

TABLE III

ACTIVATION OF IAA OXIDASE BY 2,4-DICHLOROPHENOL IN THE PRESENCE AND ABSENCE OF Mn^{++}

ENZYME PREPARATION, μGM PROTEIN	Mn^{++} (MICROMOLES)	2,4-DICHLOROPHENOL PRESENT, MICROMOLES				
		0.75	1.5	3.0	6.0	12.0
		μl O_2 uptake after 90 min				
50	...	29	29	27	13	...
100	...	76	80	57	45	...
150	...	79	92	79	31	...
50	3.0	79	183	260	261	239
100	3.0	160	277	404	371	296
150	3.0	239	369	374	341	255

micromoles of Mn^{++} supported the same oxygen uptake; therefore, 3 micromoles of Mn^{++} was used in the standard test system. Although the total oxygen uptake was the same throughout the range between 0.3 and 30 micromoles, the initial rate was greater at the higher levels, as indicated by the higher $Q_{O_2}(N)$ values which could be calculated. No advantage was gained by using higher levels of Mn^{++} since managnese phosphate precipitated.

Ferric (1) and Co^{++} (2) ions have been reported to be inhibitors of the IAA oxidase system from etiolated pea epicotyls. In the crude lupine system they may show this effect, but neither was inhibitory to-

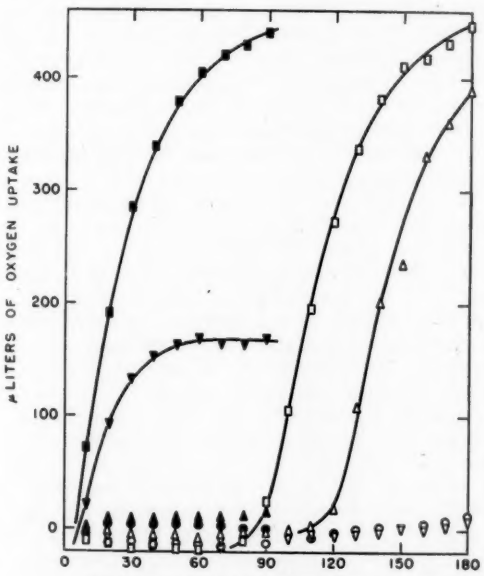


FIG. 4. Effect of dialysis on the IAA oxidase inhibitors present in lupine leaf preparations: ○, enzyme; △, enzyme + Mn^{++}; ▽, enzyme + 2,4-dichlorophenol; □, enzyme + Mn^{++} + 2,4-dichlorophenol. Open symbols, centrifuged crude enzyme extract; filled symbols, dialyzed and recentrifuged enzyme extract.

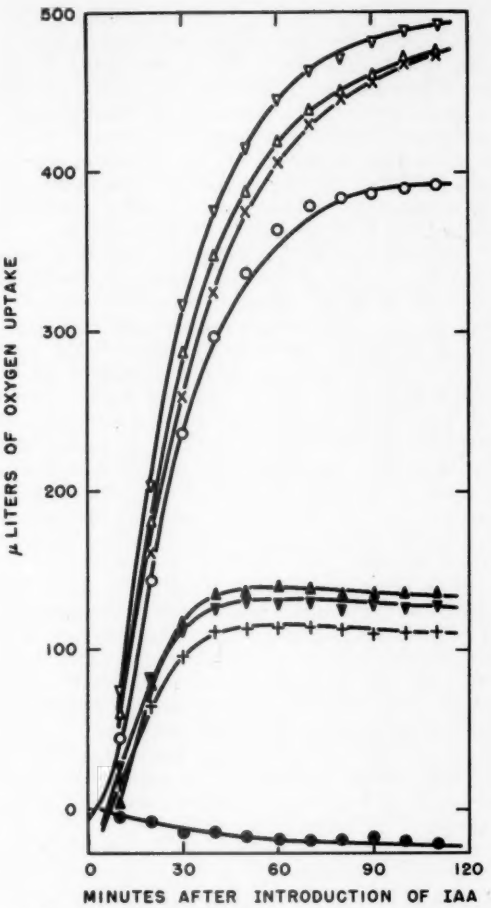


FIG. 5. Effect of metal ions on IAA oxidase. The enzyme was purified by ammonia sulfate fractionation. Each flask contained 3 micromoles of 2,4-dichlorophenol and 3 micromoles of metal ion: +, control; ×, Mn^{++}; ●, Cu^{++}; ○, Cu^{++} + Mn^{++}; ▲, Co^{++}; △, Co^{++} + Mn^{++}; ▼, Fe^{+++}; ▽, Fe^{+++} + Mn^{++}.

wards the purified enzyme; on the contrary, they were slightly stimulatory in the presence of Mn^{++}.

Cupric ion (1), however, inhibited both the crude and the purified enzymes; it appeared to block the oxidase portion of the system, but it had only slight effect on the peroxidase activity. Magnesium and calcium ions were apparently without effect.

Table I shows the distribution of activity following fractionation. The disparity and the lack of clearly defined activity distribution suggests that variations of the enzyme may exist in the plant or they may result from the disintegration procedure, perhaps by adsorption of active material on inert carrier substances. This latter hypothesis was substantiated in part by the fact that activity moved at the same rate during electrophoresis regardless of the tissue source and pH, whether etiolated or non-etiolated.

The data presented in table I seem to show greater purification of the IAA oxidase than of the purpurogallin peroxidase activity. The difference may be apparent only because inhibitors of IAA oxidase are lost on purification; furthermore, crude plant extracts may contain several peroxidases some of which would be lost during the purification (6). These considerations as well as the electrophoretic homogeneity and the absence of other peroxidases (see fig 1) constitute the principle basis for assuming a single protein.

ELECTROPHORESIS: The failure to separate the oxidase and peroxidase activity electrophoretically is illustrated in figure 1, which shows the coincidence of both activities with a single protein peak. The purification at pH 5.5 in 0.025 M acetate buffer gave better resolution than was usually achieved in orthophosphate buffer at pH 7. The PZ′ values of 1100 and $Q_{O_2}(N)$ of 2×10^5 were the highest obtained. The electrophoretic inseparability of the oxidase and peroxidase activities leads to the conclusion that they are part of the same protein molecule. This conclusion is supported also by the fact that the enzyme showed the same migration characteristics between pH 5.5 and 8.5.

EFFECTS OF INHIBITORS: Citrate buffer was inhibitory to the IAA oxidase system. Its interaction with Mn^{++} was indicated because the inhibition occurs in the presence, but not in the absence of added Mn^{++} (when the partially purified enzyme is used). The obligate requirement for the addition of a phenol was shown with citrate as with orthophosphate. The organic acid buffers, succinic and malic acid, exerted different degrees of inhibition, suggesting that the inhibitory activity was dependent on the binding capacity of the anion for Mn^{++} (13).

Pyrophosphate is reported to be inhibitory to horseradish peroxidase (6). With the purified lupine enzyme, however, pyrophosphate buffer was only partially inhibitory (about 30 %) when the enzyme was acting as a peroxidase on pyrogallol and H_2O_2, but totally inhibitory when IAA was used as the substrate in the presence of 2,4-dichlorophenol with or without added Mn^{++}.

Although the addition of glycolic acid shortened the lag caused by the naturally occurring inhibitors present in some of the crude enzyme preparations; it was inhibitory towards the pure enzyme. Since H_2O_2 may be formed by the glycolic acid oxidase (23) which is present in these crude enzyme preparations, either H_2O_2 or a peroxide radical is being used by the peroxidase portion of the enzyme to remove the naturally occurring inhibitors of the IAA oxidase system. An alternative would be that H_2O_2 primes the oxidation of IAA via the peroxidase.

Glucose plus glucose oxidase can also be coupled to the oxidase system via peroxide radical formation. This was done with the purified enzyme giving rise to optimum substrate utilization in the presence of 2,4-dichlorophenol and in the absence of Mn^{++}. At substrate levels, 30 micromoles of H_2O_2 appears neither to be used nor grossly to affect the reaction. Instead it was rapidly decomposed as if catalase was present.

The classical inhibitors for heme enzymes were tested with both the oxidase and peroxidase systems (table IV). The difference in sensitivity of the two systems was probably more apparent than real, since it was roughly equal to the difference in enzyme concentration used.

The carbonyl-trapping agents were initially investigated in an attempt to trap indole-3-carboxaldehyde if it were formed during the reaction. The oxidase reaction was found to be inhibited by the concentrations required for trapping, while the peroxidase system was relatively insensitive as is indicated for semicarbazide. Since there seems to be no aldehyde trapping or effective heme inhibition by the semicarbazide, the mechanism of its action on the oxidase is not clear.

Cyanide showed the same pattern of response as azide, and the inhibition of the oxidase was easily reversed by evaporation of the gas. The oxidase was quite insensitive to sulfide while the peroxidase was very sensitive.

Kenten (6) effectively used the inhibition of horseradish peroxidase by catalase in the study of the mechanism of the peroxidase action on IAA, and Galston has reported a similar response for IAA oxidase (1). In contrast, the IAA oxidase from lupine was not inhibited by the presence of catalase either at microgram or milligram levels, nor was it affected at all by light or riboflavin. In fact, catalase usually exerted a slightly stimulatory action.

SUBSTRATE SPECIFICITY: Although some crude enzyme preparations are capable of using indole-3-propionic and indole-3-butyric acids at much lower levels of efficiency than IAA, the purified enzyme used indole-3-propionic acid at 14 % the efficiency at which it used IAA, and showed no oxygen uptake on indole-3-butyric acid. β-Indoleacetonitrile, 5-hydroxy-IAA, indole-3-carboxaldehyde, tryptophan and naphthalene acetic acid did not support oxygen uptake with the purified enzyme. Significantly the system appears to be incapable of using added H_2O_2 in the oxidation of IAA, but it can use enzymatically generated peroxide or peroxide radical in the presence of 2,4-dichlorophenol as was found with coupled glucose oxidase.

TABLE IV

EFFECT OF INHIBITORS

INHIBITOR	MOLARITY OF INHIBITOR RESULTING IN 50 % INHIBITION	
	OXIDASE *	PEROXIDASE
NaN_3	1×10^{-3}	5×10^{-5}
NaCN	5×10^{-5}	1×10^{-4}
$(NH_4)_2S$	5×10^{-3}	1×10^{-5}
NH_2OH	5×10^{-4}	7×10^{-4}
Semicarbazide	5×10^{-5}	7×10^{-3}

* The amount of enzyme used in the oxidase systems was ten times higher than that used in the peroxidase determination.

PRODUCTS OF THE IAA OXIDASE REACTION: The reaction products were studied by paper chromatography of the reaction mixture from the Warburg flasks on Whatman 3 mm paper; the solvent system was 1 : 1 : 10 28 % NH_4OH : water : isopropyl alcohol (v/v). Ascending development was found to give better separation than the descending method. The results are given in table V. Examination of the chromatograms under ultraviolet light, and spraying with a modified Salkowski reagent (4), revealed the formation of 5 or 6 products, two of which were non-indolyl in nature; this conclusion was substantiated by ultraviolet spectrophotometric examination of eluates from the chromatograms. In corresponding experiments with the reaction mixture in the absence of IAA no spots could be detected on the paper.

Indole-3-carboxaldehyde (12) could not be identified among the usual products of the reaction. The examination of the reaction products was impeded by the formation of dark, apparently polymerized material which was insoluble in water and ether. Although 3-indolecarboxaldehyde appears to be normally absent, it could constitute a major component of the polymerized fraction.

When the reaction mixture was adjusted to pH 10 and extracted with ether, then adjusted to pH 3 and again extracted with ether, three components were found in the basic-neutral fraction and four were found in the acid fraction (table V). Nothing could be detected in the aqueous phase after the insoluble polymeric indolyl material was filtered off. Ultraviolet examination with the Cary automatic spectrophotometer confirmed the indolyl nature of three of the 6 fractions; the 290 and 280 $m\mu$ peaks of the indole components were present.

The use of carboxyl-labeled IAA showed that decarboxylation is almost quantitative if an RQ of 1 is assumed. However, neither radioautography nor direct counting of the chromatograms has given a clearly defined picture of the non-carbonate radioactivity. Valid data could not be obtained because of the general distribution of the low levels of C^{14} activity on the chromatograms derived from the polymerized material.

TABLE V

PAPER CHROMATOGRAPHY OF IAA OXIDASE REACTION PRODUCTS

R_f	Reaction mixture untreated		Soluble in ether at pH 10		Soluble in ether at pH 3	
	U.V.*	Salkowski	U.V.*	Salkowski	U.V.*	Salkowski
0.3	1	IAA	..	.....	1	IAA
0.45	..	Indolyl	..	.....	4	Indolyl
0.6	2	Non-indolyl	..	.....	2	Non-indolyl
0.7	4	Non-indolyl	4	Non-indolyl	4	Non-indolyl
0.8	1+4	Indolyl	1	Indolyl	4	Non-indolyl
0.9	3	Indolyl	3	Indolyl	..	.....

*U.V. response: 1, dark zone, U.V. absorbing material; 2, green fluorescence; 3, red fluorescence; 4, white fluorescence.

DISCUSSION

The ammonium sulfate and electrophoretic fractionation of the IAA oxidase system from lupine has resulted in a purification of about 1000 times (table I). Since the oxidase and peroxidase activities were found to be inseparable (fig 1), it is not surprising that a reduction in the number of products has not been achieved through this approach.

The amphoteric nature of the enzyme and its ability to attach loosely to inert proteins are indicated by the apparent variability in solubility of enzyme from different tissue sources in ammonium sulfate (table I), and the electrophoretic mobility of the enzyme, regardless of pH. The data suggest that the enzyme is a relatively small protein with a weak positive charge.

With purification there is a simultaneous loss of both the natural activator and inhibitor (fig 4); the activator can be replaced in part (at a lowered level of efficiency) by the addition of a phenol (i.e., 2,4-dichlorophenol). This is consistent with the observations of others (3, 6, 21) who have used pea, bean root, horseradish, and wheat as sources of the enzyme. The importance of the Mn^{++} is emphasized by the fact that inhibition by organic acids (6) seems to be dependent on the Mn^{++}-binding capacity (13) of the anion. Citrate and pyrophosphate, being better chelators for Mn^{++} than orthophosphate, may exert their inhibitory effect by binding the active enzyme-Mn^{++} complex. The Mn^{++} inhibition previously reported by Galston et al (1) was undoubtedly caused by the citrate buffer used.

The removal of the natural activator and inhibitor is accompanied by a shift in the pH optimum (fig 2). In fact, if 30-, 60-, 90-, and 120-minute plots of the pH response of the crude enzyme are made, a shift of the optimum toward the basic side is usually found which in part accounts for the overcoming of the inhibitor-induced lag with time. Since the inhibition is slowly reversed at higher pH levels, the pH 6.3 optimum is quite common in such preparations; this is in agreement with the observation of Wagenknecht and Burris (20) for the enzyme from the wax bean root. Since both the activator and inhibitor are ether-soluble and heat-stable as well as dialyzable, it may be concluded that they are small organic molecules, perhaps phenolic in character.

The obligate requirement for addition of a phenolic activator is clearly shown in figures 3 and 4. The differences in activity with variations in the levels of enzyme and 2,4-dichlorophenol in the presence or absence of Mn^{++} are illustrated in table III. In the absence of Mn^{++}, oxygen uptake is more dependent on the enzyme concentration than on the level of 2,4-dichlorophenol. In the presence of Mn^{++}, the increase

in oxygen uptake varies both with the amount of enzyme and with the amount of 2,4-dichlorophenol (up to 6 micromoles of the phenol). At 0.75 micromoles of 2,4-dichlorophenol, the changes are strictly dependent on the enzyme concentration. Neither Mn^{++} nor 2,4-dichlorophenol will support oxygen uptake in the absence of IAA.

The specificity of the requirement of the lupine enzyme for Mn^{++} is shown by the failure of other cations, particularly Fe^{+++} and Co^{++}, to replace Mn^{++}. These metal ions do not elicit the inhibitory responses reported for the pea system (1, 2). As with the observations on the pea enzyme (1) copper was found to be inhibitory in either the presence or absence of Mn^{++}. A similar response to Cu^{++} has been found by Knox (7) using tryptophan peroxidase.

The secondary nature of the requirement for added Mn^{++} is substantiated further by the fact that the activity (in the presence of 2,4-dichlorophenol) is the same in citrate or orthophosphate buffer without added Mn^{++}, but is totally inhibited in citrate buffer when Mn^{++} is added. Possibly an enzyme-Mn^{++} complex is removed from solution by chelation by the citrate ion, since neither citrate nor Mn^{++} alone is inhibitory.

In the absence of added Mn^{++}, activation can be brought about by coupling the system to a peroxide generating system, e.g., glucose oxidase. This indicates that one of the following pathways may be functioning: first, since the peroxidase portion of the system can be activated in the presence of 2,4-dichlorophenol, the glucose oxidase may be replacing Mn^{++} in the generation of the peroxide radical. Second, the inability of enzyme alone to use enzymatically generated H_2O_2 in the absence of 2,4-dichlorophenol (but in the presence of added Mn^{++}) suggests that a dehydrogenase may first act upon IAA and that it requires a phenolic acceptor. Third, some of the Mn^{++} when added may be oxidized in the manner suggested by Kenten (6) or Waygood (21). However, in spite of the enhanced degree of activation, the ineffectiveness of Mn^{++} alone or Mn^{++} plus H_2O_2 argues against this. The inability of catalase to block the reaction may be caused by the fact that the concentration of peroxide radicals is too low or that they are in a form unavailable to the catalase. This leads to the conclusion that a dehydrogenase-like portion of the electrophoretically homogeneous protein molecule is coupled to the peroxidase by a phenol or phenolic radical, the formation of which is stimulated by added Mn^{++}.

The high order of substrate specificity to argue against the action of IAA as a reducing agent for the cyclic operation of a peroxidase as has been suggested by Kenten (6). The failure to demonstrate the action of the dehydrogenase by coupling with the usual hydrogen transport agents suggests that it may normally act via a phenol in the intact plant. There is no evidence for the involvement of a light-activated flavoprotein, since the enzyme from etiolated plants grown, prepared, and tested in the dark is as active as the enzyme from the same source prepared and tested in the light.

The multiplicity of the products and the apparent failure to reduce their number by purification of the enzyme or by the use of inhibitors is good evidence for the multiple nature and complexity of the oxidase system. The failure to detect indole-3-aldehyde, even in the amounts reported by Racusen (12), does not preclude the possibility that it is formed or is a transitory intermediate in the system as studied.

In contrast to earlier reports, this study shows that all parts of the etiolated or non-etiolated *Lupinus albus* L. may be used for the preparation of the IAA oxidase. In many respects the enzyme from this source resembles the IAA oxidase obtained from other sources. There are, however, several differences, and it remains to be seen whether these are real or apparent only because of differences in purity and method of preparation. Until more information is at hand it would seem desirable to avoid generalizations concerning composition and mode of action of the IAA oxidases from different sources.

Summary

The indole-3-acetic acid oxidase-peroxidase from *Lupinus albus* L. has been purified by $(NH_4)_2SO_4$ fractionation and electrophoresis. The electrophoretically homogeneous enzyme was found to form the same products as crude enzyme obtained from the aerial parts of either etiolated or non-etiolated lupine. The enzyme shows an obligate requirement for a phenolic activator and is stimulated by added Mn^{++} in orthophosphate buffer. Citrate and pyrophosphate inhibition appears to be dependent on the Mn^{++}-chelating ability of the anion. The inhibitory action of the heme inhibitors and carbonyl-trapping agents was examined for both the oxidase and peroxidase activities. The carboxyl carbon of indoleacetic acid is found as CO_2; the fate of the rest of the molecule has not been established. Indole-3-aldehyde is not formed in detectable quantities.

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SOME EFFECTS OF GIBBERELLIN ON FLOWERING AND FRUIT SETTING[1,2,3]

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Some remarkable growth stimulatory effects of gibberellin (GB) on dwarf type plants have been reported (1, 2, 8). While the hyperelongation of internodes, and increase in fresh weight and dry matter are easily identified, little if any attention has been directed to the effects on earliness of flowering and other allied reproductive responses. The potential offered by these substances in control of plant growth may not only be useful in promoting greater productivity of crops grown for their vegetation, but for earlier maturity and greater yields of fruit and seed crops.

PARTHENOCARPY IN TOMATOES: The comparative effectiveness (10), as percentage of fruit set, of indole-3-acetic acid and GB applied in lanolin at different concentrations for induction of parthenocarpy in tomato ovaries is presented in table I. Whereas the comparisons are relative, the greater effectiveness of GB at very low concentrations is apparent, and equals or exceeds that of any known indole compound (10). This is one additional bio-assay in which the effects of GB on growth resemble those of auxin (3, 4, 7).

FLOWERING IN BEANS: Three varieties of beans differing in maturity and growth habits, consisting of Contender (early determinate), Rival (midseason-semi-determinate) and Blue Lake (late-indeterminate) were seeded simultaneously and the apex following expansion of the primary leaves treated by applying 0.01 ml of water solution containing the designated micrograms of GB (table II). Flowering in Contender, an early market green bean, occurred in significantly fewer days as a result of treatment irrespective of the amount of GB applied. A significant response not only in earlier flowering but in greater vegetative growth was apparent (fig 1). Differences in time of flowering for Rival and Blue Lake were not

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[2] Journal Article No. 1948 from the Michigan Agricultural Experiment Station.
[3] The gibberellin used in this study consisted of a mixture of gibberellins A and X (gibberellic acid) having a rotation of $[\alpha]_D + 62$, supplied by Dr. F. H. Stodola, Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Illinois.

TABLE I

RELATIVE EFFECTIVENESS OF INDOLE-3-ACETIC ACID (IAA) AND GIBBERELLIN (GB) IN LANOLIN ON THE STIMULATION OF PARTHENOCARPY IN THE TOMATO

CONC OF IAA AND GB IN LANOLIN (%)	VARIETY OF TOMATO			
	FIREBALL		MICHIGAN-OHIO HYBRID	
	IAA	GB	IAA	GB
10^{-1}	+++*	+++	+++	+++
10^{-2}	+++	+++	++	+++
10^{-3}	Inactive	+++	Inactive	++
10^{-4}	"	+	"	+
10^{-5}	"	+	"	+
10^{-6}	"	Inactive	"	Inactive
Lanolin (control)	Inactive	Inactive	Inactive	Inactive

* The number of plus signs at each concentration indicates the relative magnitude of activity of each substance.

significant although marked hyperelongation of internodes occurred.

FLOWERING IN TOMATOES: Five varieties of tomatoes varying in growth habit from the early–extremely dwarfed PI-205046 to the late–highly indeterminate Rutgers (table III) were treated as were the beans, when the first true (plumule) leaf was emerging. The comparative number of days to first anthesis, and flower number 52 days after seeding, are listed in table III for plants receiving 0, 10 and 20 micrograms of GB. As with beans earlier and more prolific flowering occurred with the early dwarf or strongly determinate varieties PI-205046 (fig 2) and Early Chatham. The earlier flowering was correlated with a more rapid growth and not with a reduction in node number preceding the first flower. There was no effect on Moreton Hybrid, Stokesdale or Rutgers, all strongly indeterminate varieties.

STEM (SEEDSTALK ?) ELONGATION: Marked stem elongation in lettuce and cabbage plants occurred within 5 to 7 days following treatment of the apices with 5 to 20 micrograms of GB. Leaf lettuce (vars. Grand Rapids, Bibb and Tendergreen) treated at the 10 to 12 leaf stage flowered 3 to 5 days earlier and seedstalks grew twice as tall. Head lettuce grown under conditions conducive to heading, and treated at various stages of growth prior to and during early head formation, subsequently bolted, formed no heads (fig 2), and inflorescence primordia became visible fully four weeks earlier than on plants not treated. GB treated plants subsequently flowered and produced viable seed 31 days earlier than the controls. Reproductive development was essentially the same as that which occurs with plants grown from vernalized seed and exposed to high temperatures and a long photoperiod (9).

TABLE II

DAYS TO THE APPEARANCE OF THE FIRST FLOWER IN SNAP BEANS AS INFLUENCED BY GIBBERELLIN

VARIETY	GIBBERELLIN (μGM/PLANT)			
	0	5	10	20
Contender	30.2	27.2	27.2	27.2
Rival	32.4	31.0	31.8	32.0
Blue Lake	34.2	33.4	33.8	34.2
Conc means (all varieties)	32.3	30.5	30.9	31.1

Least differences necessary for significance between concentration means: 5% = 0.6, 1% = 0.8.

FIG. 1. Comparative vegetative and flowering response of the bean (var. Contender) 31 days from seeding. *Left.* Control (no treatment). *Right.* Apex treated with 20 μgm of gibberellin after expansion of the primary leaves.

Similarly when the apices of young (6 to 8 leaf stage) cabbage plants (vars. Golden Acre and Danish Ballhead) were treated with 20 micrograms of GB and the application repeated at weekly intervals, no heads formed and the stems elongated as if highly reproductive (6) but no flowering occurred. The requisite cold treatment was not replaced by GB as has been reported for Hyoscyamus (5), although plants appeared fully reproductive, other than that no flowers or flower primordia were formed. With both head lettuce and cabbage, the firm head which often forms a mechanical barrier was eliminated and seedstalks formed rapidly.

TABLE III

EFFECTS OF GIBBERELLIN ON FLOWERING IN THE TOMATO AS RELATED TO TIME FROM SEEDING

VARIETY	DAYS FROM SEEDING TO FIRST ANTHESIS			NO. OF FLOWERS/PLANT, 52 DAYS AFTER SEEDING		
	0	10	20	0	10	20
	μgm gibberellin/plant					
PI-205046 * (very dwarf)	37.4	32.6	34.8	75.8	79.2	119.2
Early Chatham	39.0	31.8	33.0	44.4	49.0	60.0
Moreton Hybrid	41.4	41.2	37.6	21.4	21.4	19.8
Stokesdale	41.8	41.2	38.8	16.6	20.8	19.8
Rutgers	44.0	44.2	45.4	15.4	10.6	11.6

Least differences necessary for significance between gibberellin concentrations within any variety for days to first anthesis: 5 % = 3.6, 1 % = 4.7; for flower number: 5 % = 7.0, 1 % = 9.3.

* Seed supplied by M. M. Hoover, Regional Plant Introduction Station, Horticultural Crops Research Branch, Ames, Iowa.

SUMMARY

Parthenocarpic fruit development in the tomato and earlier flowering in several plants was induced by treatment with gibberellin. For induction of parthenocarpy the response to gibberellin was similar to that of indole-3-acetic acid but effective concentrations extended below those reported for any indole compound. Earlier flowering in crops that responded to GB occurred from a pronounced acceleration of vegetative growth, and in the case of head lettuce the elimination of the head as a mechanical barrier. Gibberellin did not specifically influence the flowering process since the amount of vegetative growth expressed as leaf numbers preceding the first flowers was not altered.

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FIG. 2. The effect of gibberellin on the vegetative and reproductive responses of head lettuce (var. Great Lakes) and the tomato (PI-205046). *Upper, left*—Control (no treatment); *right*, apex of lettuce plant treated with 10 μgm of gibberellin 20, 13 and 6 days prior to photographing. *Lower, left*—Control (no treatment); *center*, 10 and *right*, 20 μgm of gibberellin applied at the emergence of the first true (plumule) leaves.

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EFFECTS OF HERBICIDES AND RELATED CHEMICALS ON OXIDATION AND PHOSPHORYLATION BY ISOLATED SOYBEAN MITOCHONDRIA [1,2,3]

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Herbicides have been shown to affect many diverse processes in plants. Respiratory responses, however, are general, and the idea that a common site of action exists somewhere in the oxidation or phosphorylation systems would seem to be a good working hypothesis (11). In recent years evidence has accumulated that the enzymes involved in aerobic plant respiration and phosphorylation are located in discrete cytoplasmic particles called mitochondria (5, 8). The reactions mediated by these enzymes may be carried out in vitro by properly isolated particles.

Although isolated plant particles would appear to be favourable for the study of the effects of herbicides on respiratory enzyme systems, only one reference (3) to such an investigation has been found. The experiments reported here were carried out with an in vitro particulate system with the hope of further elucidating the mechanism of action of certain herbicides and related chemicals in vivo.

The particles used were isolated by differential centrifugation from etiolated soybean hypocotyls and were shown to have oxidative and phosphorylative activities similar to those from mung beans (10). In most experiments treatments consisted of the addition of various concentrations of herbicide or other chemical to Warburg flasks containing the reaction mixture. The effect of the chemical on oxygen uptake by the soybean particles was then expressed as percentage of control (table I). Some of the chemicals tested inhibited oxygen uptake only when added at relatively high concentrations. This suggests an osmotic effect, and probably such an explanation is correct for chemicals that inhibited only at concentrations of 0.1 M or higher, such as sodium chlorate (cf. sodium chloride, table I). Others including 2,4-dichlorophenoxyacetic

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TABLE I

EFFECT OF VARIOUS CHEMICALS ON OXYGEN UPTAKE BY ISOLATED SOYBEAN PARTICLES *

CHEMICAL	SUBSTRATE	RANGE OF CONC TESTED (M)	CONC TO BRING ABOUT 50 % INHIBITION OF O_2 UPTAKE (M)	MAX INHIBITION (%)
2,4-Dichlorophenoxyacetic acid	Succinate	10^{-2} to 10^{-6}	10^{-3}	78
	Pyruvate	10^{-2} to 10^{-6}	2×10^{-3}	92
	Ketoglutarate	10^{-2} to 10^{-6}	5×10^{-3}	80
2,4-Dichlorophenol	Succinate	7.5×10^{-4} to 10^{-6}	5×10^{-4}	69
	Pyruvate	7.5×10^{-4} to 10^{-6}	2×10^{-4}	100
	Ketoglutarate	7.5×10^{-4} to 10^{-6}	2×10^{-4}	80
Indoleacetic acid	Succinate	5×10^{-3} to 10^{-9}	.....	27
	Pyruvate	5×10^{-3} to 10^{-9}	.....	12
2,4,5-Trichlorophenoxyacetic acid	Succinate	5×10^{-3} to 10^{-7}	10^{-3}	82
	Pyruvate	5×10^{-3} to 10^{-7}	10^{-3}	88
2,2-Dichloropropionic acid	Succinate	10^{-1} to 10^{-6}	5×10^{-2}	78
	Pyruvate	10^{-1} to 10^{-6}	5×10^{-2}	64
Sodium chlorate	Succinate	5×10^{-1} to 10^{-6}	2×10^{-1}	73
	Pyruvate	5×10^{-1} to 10^{-6}	.....	25
Sodium chloride	Succinate	5×10^{-1} to 10^{-5}	10^{-1}	90
	Pyruvate	5×10^{-1} to 10^{-5}	5×10^{-1}	50
Trichloroacetic acid	Succinate	10^{-1} to 10^{-6}	2×10^{-2}	85
	Pyruvate	10^{-1} to 10^{-6}	5×10^{-2}	91
Maleic hydrazide	Succinate	10^{-2} to 5×10^{-6}	.....	9
	Pyruvate	10^{-2} to 5×10^{-6}	.....	25
2,3,5-Triiodobenzoic acid	Succinate	1.25×10^{-3} to 10^{-6}	2×10^{-4}	86
Coumarin	Succinate	2×10^{-3} to 10^{-5}	.....	3
	Pyruvate	2×10^{-3} to 10^{-5}	.....	2

* All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose 0.2 M; phosphate 0.005 M. Herbicide or other chemical being tested was added directly to reaction mixture.

TABLE II

EFFECT OF 2,4-D ON OXIDATIVE PHOSPHORYLATION BY SOYBEAN PARTICLES WITH SUCCINATE AS SUBSTRATE *

2 4-D (M)	O_2 UPTAKE **	P UPTAKE **	P : O
Control	13.5	12.8	0.95
5×10^{-5}	13.4	12.3	0.92
1×10^{-4}	12.0	11.8	0.98
5×10^{-4}	10.4	8.5	0.82
1×10^{-3}	6.6	3.5	0.53
5×10^{-3}	3.6	1.3	0.36

* All flasks contained substrate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; glucose, 0.1 M; sucrose, 0.1 M; phosphate, 0.00625 M; hexokinase, 2.5 mg.

** Oxygen uptake expressed as microatoms/hr per flask, and phosphate uptake as micromoles/hr per flask.

acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid, were inhibitory down to 5×10^{-5} M. Inhibition in these cases cannot be attributed to a change in the tonicity of the reaction medium. The toxic action of most of the chemicals is apparently not specific, since oxygen uptake was affected to approximately the same extent in the presence of all substrates tested. It would appear, therefore, that this inhibited oxygen uptake is brought about by a general toxic action of the chemicals on the whole enzyme complex rather than on individual enzymes. A similar conclusion was reached by Dow (3) on the basis of his results with growth substances on isolated lupine mitochondria.

No consistent increase in respiration was brought about by any concentration of the chemicals that were tested, except for 2,4-D which did increase oxygen uptake if no inorganic phosphate was added to the reaction mixture. Under these conditions, the systems to which 2,4-D was not added had a low rate of respiration (about 100 μl/hr × mg N with ketoglutarate as substrate) and stimulations of 15 to 20 % were consistently obtained with 10^{-4} M 2,4-D. Since the supply of inorganic phosphate in the reaction vessels containing 2,4-D increased during these experiments (from 0.3 to 1.1 micromoles per flask in a typical experiment in which 10^{-4} M 2,4-D was added) while that of the control flasks did not increase appreciably, it would appear that this herbicide was acting as an uncoupling agent. In intact tissue, 2,4-D is known to behave as an auxin, and as such will stimulate respiration and growth under appropriate conditions (1). One must, of course, consider possible differences in the concentration of 2,4-D at the site of action in vivo and in vitro. Whether this herbicide acts to stimulate respiration by promoting growth or by acting as an uncoupling agent may largely depend on the concentration present.

Since several investigators have indicated that a close relationship exists between the action of 2,4-D and changes in respiration (1, 7, 9), this herbicide was investigated more extensively than any of the other chemicals. The effects of 2,4-D on in vitro oxidation and phosphorylation by soybean particles are presented in table II. Both oxygen and phosphate uptake were markedly inhibited by concentrations of 2,4-D of 5×10^{-4} M and higher, with phosphate uptake showing the greater effect. This resulted in a depression of the P : O ratio at these levels of 2,4-D treatment. This response was similar to that brought about by dinitrophenol or by dichlorophenol but was not as marked. For example, 5×10^{-5} M DNP or 10^{-4} M DCP decreased oxygen uptake 15 to 25 % and decreased phosphate uptake 50 to 75 % resulting in a marked depression of the P : O ratio. The possibility that the 2,4-D effect was caused by contamination with dichlorophenol (6) was investigated. Analysis of the 2,4-D used showed that the quantity of phenol present (0.04 %) was too low to be responsible for any of the effects of the 2,4-D. It would appear that the enzymes involved in phosphorylation in vitro were more severely affected by 2,4-D than those involved in oxidation. These results are similar to those obtained by Brody (2) using animal mitochondria and support the hypothesis of Bonner and Bandurski (1).

In addition to these experiments in which 2,4-D was added to the reaction mixture, the effect of spraying etiolated plants with 2,4-D before isolation of the particles was also studied. Table III shows the results of representative experiments of this type, in which soybeans were sprayed with 5×10^{-4} M 2,4-D at 24, 18, and 12 hours prior to harvest. The 24-hr and 18-hr treatments both increased the activity of particles in relation to those from untreated plants of the same age. The results from the 12-hr treatment were variable. In general, phosphate uptake was increased to about the same extent as oxygen consumption, although considerably more was consumed in the reaction mixture containing particles

TABLE III

EFFECT OF PRETREATMENT OF SOYBEAN HYPOCOTYLS WITH 0.0005 M 2,4-D AT VARIOUS TIMES BEFORE HARVEST ON THE RESPIRATION AND PHOSPHORYLATION OF ISOLATED CELL PARTICLES *

TREATMENT, HRS BEFORE HARVEST	SUBSTRATE	% CONTROL **		
		O_2 UPTAKE	P UPTAKE	P : O
24	Succinate	149	155	104
	Pyruvate	135	172	127
	Ketoglutarate	120	137	114
18	Succinate	132	133	100
	Pyruvate	137	156	114
	Ketoglutarate	134	106	79
12	Succinate	127	220	173
	Pyruvate	77	82	107
	Ketoglutarate	99	156	158

* All flasks contained substrate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; glucose, 0.1 M; sucrose, 0.1 M; phosphate, 0.00625 M. Malate, 0.0017 M, added with pyruvate.

** Activity of particles from treated plants expressed as percentage of the activity of particles from untreated plants of the same age and isolated at the same time (on a nitrogen basis).

from the 12-hr treated seedlings with succinate or ketoglutarate as substrate.

The question arises whether 2,4-D changed the activity of the particulate enzymes in vivo, or whether the increased activity may have been related to changes in the morphology of the tissue as a whole. All sprayed plants were swollen and turgid at time of harvest, with the degree of such effects depending upon length of time since spraying. Perhaps particles from this turgid tissue did not lose as much activity during isolation, or inhibitors were present in smaller quantities. Some support for this idea was provided by the fact that endogenous oxygen uptake was also more rapid in the experiments in which seedlings were pretreated with 2,4-D, and also by the results of experiments in which dichlorophenol (10^{-5} M) was sprayed on soybean hypocotyls 24 hours before isolation of the particles. This treatment with dichlorophenol brought about no change in the external appearance of the plants and decreased oxygen uptake of the isolated particles slightly (10 %) in relation to the control. If the effects of 2,4-D shown in table III were brought about by changes in the particulate enzyme system in vivo, similar results would be expected with dichlorophenol which has been shown to act in much the same way as 2,4-D on the respiration of intact tissue (4). It would appear, therefore, that the increased oxidative and phosphorylative activity of particles isolated from soybean hypocotyls sprayed with 2,4-D was due to formative effects of this chemical leading to isolation of particles that retained more of their original activities.

Summary

Although the site of action of none of the chemicals tested was pin-pointed, a general inhibition of the respiratory enzymes of isolated soybean particles was demonstrated. Of the chemicals that were studied, those known to have growth regulating properties when applied to intact plants were the more active respiratory inhibitors in vitro.

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THE HEXOSE MONOPHOSPHATE SHUNT AS THE MAJOR RESPIRATORY PATHWAY DURING SPORULATION OF RUST OF SAFFLOWER [1,2]

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Infection by obligate plant parasites characteristically causes large increases in respiratory rates and in the cases investigated there is observed an inhibition of the Pasteur effect found in normal uninfected tissue (1, 2). Since the host tissue appears to mediate the higher rates of respiration (3, 10), it has been suggested that the respiratory increases and the inhibition of the Pasteur effect result from the action of naturally occurring uncoupling agents (1, 7).

Hypocotyl infection of safflower, *Carthamus tinctorius* L., by the rust, *Puccinia carthami* Cda., induces similar changes in respiratory behavior and also causes an increase in host growth during mycelial growth of the parasite (5), a feature inconsistent with

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the presence of uncoupling toxins. During sporulation of the parasite, however, host growth ceases and the high rates of respiration then are associated with a marked reduction in the magnitude of the Pasteur effect. This paper describes some of the aspects of the phenomenon in sporulating tissue of the host-parasite complex and shows that the inhibition of the Pasteur effect in this instance is a consequence of the replacement of the major respiratory mechanism of normal hypocotyl tissue by the direct oxidative pathway of glucose metabolism, the hexose monophosphate shunt (4).

Methods

Inoculation and growth of the host-parasite complex were as previously described (5). Several different lots of plants were utilized and since the amount of infection in the various experiments could not be controlled, quantitative comparisons of data among different lots cannot be made. Absolute respiratory values, even for healthy plants, show some variation because light intensity and day length varied.

Hypocotyl sections 2 to 3 mm in length were floated on 0.1 M KH_2PO_4 for respiratory measurements in standard Warburg equipment. Five hundred mg of sections were used in duplicate vessels, after randomization of tissue. Aerobic CO_2 was determined by Warburg's direct method and anaerobic CO_2 was measured in N_2 of 99.9 % purity. All data are expressed as microliters gas exchange per gram fresh weight.

Sodium fluoride or 2,4-dinitrophenol (DNP) were added one hour before initial readings to allow uniform penetration and the establishment of uniform rates. In studying the effect of these materials on anaerobic CO_2 production, N_2 was flushed through the vessels for 15 minutes immediately after addition of the chemicals, permitting 45 minutes for equilibrium of the gas. Transient outbursts of CO_2 were noted during equilibration, but were not measured since we were interested only in steady state conditions.

In the tracer experiments, 25 micromoles of glucose labelled either in the first or sixth carbons was added to 2.0 ml of buffer containing 2.0 gm of tissue. The specific activities were 0.214 and 0.212 microcuries per micromole for glucose-1-C^{14} and glucose-6-C^{14}, respectively. With our assay, these represented 7.57 and 7.38×10^{-5} cpm per vessel. Since equal amounts of tissue possessing unequal respiratory activity were being compared, the time of incubation with isotopic glucose was varied so that the total CO_2 production for all experimental vessels was approximately equal. The CO_2 was trapped in 0.1 ml of 10 % KOH which was transferred in 10-lambda amounts to filter paper. Each aliquot was allowed to dry before another portion was superimposed. Several portions of distilled water were used for rinsing and were placed on filter paper in a similar manner. The filter paper was then centered under a thin-walled mica end-window Geiger tube for counting with a Tracerlab Superscaler. Counts were obtained from the side of the filter paper to which the KOH solution was added.

Results

The Pasteur Effect in Healthy and Sporulating Diseased Tissue: Table I illustrates the nature of the Pasteur effect inhibition observed in sporulating diseased hypocotyls. In normal hypocotyls, the Pasteur effect, as measured by the ratio of fermentative to oxidative CO_2 production is not as large as for many species (9), but is consistently obtained at all stages of development. In the diseased tissue, pycniospores were abundant on the 17th day and urediospore production began on the 20th day and was at a maximum by the 24th day.

During this period, the ratio of anaerobic to aerobic CO_2 production fell below 0.33 and there was an appreciable lowering of the magnitude of CO_2 of fermentation in diseased tissue; in the later stages of urediospore production it was approximately one-half of the rate of healthy tisssue.

Sodium Fluoride Inhibition of Respiration: Attempting to establish a mechanism for either the Pasteur effect or its inhibition based solely on measurement of CO_2 production is subject to uncertainties (9), in this instance chiefly because of the possibility of shifts in the final products of glycolysis (i.e., alcohol versus acid production). Associated with the decreased rates of anaerobic CO_2 production in sporulating tissue is a decrease in sensitivity of oxygen uptake to 10^{-2} M NaF. Table II presents data for two groups of plants grown under different environmental conditions. In group A, growth of both host and parasite was more rapid because of longer day length and higher light intensity but, in each instance, inhibition of oxygen uptake by NaF was approximately twice as great in healthy hypocotyls as in diseased tissue.

The data of figure 1 eliminate two possibilities for explaining the results. The total height of the bars represents the absolute values of CO_2 produced aerobically and anaerobically in the absence of inhibitor while the non-shaded areas indicate production of CO_2

Table I

The Pasteur Effect in Healthy (H) and Sporulating Rust-Infected (D) Safflower (Carthamus tinctorius L.) Hypocotyl

Days after planting	Tissue	O_2 uptake	A Aerobic CO_2	B Anaerobic CO_2	Ratio B/A
		$\mu l/gm\ fresh\ wt \times hr$			
17	H	101	110	76	0.69
	D	143	149	48	0.32
20	H	103	107	66	0.62
	D	171	178	46	0.26
24	H	104	116	74	0.64
	D	162	169	42	0.25

TABLE II

THE EFFECT OF 10^{-2} M NaF ON OXYGEN UPTAKE IN HEALTHY (H) AND SPORULATING RUST-INFECTED (D) SAFFLOWER HYPOCOTYL*

GROUP A			GROUP B		
DAYS AFTER PLANTING	% INHIBITION		DAYS AFTER PLANTING	% INHIBITION	
	H	D		H	D
17	51	33	27	45	25
20	51	30	29	59	27
24	64	32	31	40	10

* Group A plants showed more rapid development of both host and parasite and sporulation was earlier than Group B.

in the presence of 10^{-2} M NaF. The RQ of both inhibited and non-inhibited tissue was equal to unity. This fact, plus the larger % inhibition of anaerobic CO_2 production when compared to aerobic inhibition suggests that the action of NaF is, as expected, on the glycolytic mechanism and is not exerting side effects on unrecognized systems in intact tissue. In addition, the approximately equal effects of inhibitor on anaerobic fermentation in healthy and diseased tissue eliminates the possibility that differences in NaF sensitivity result from differential penetration into the two types of tissue. Thatcher has reported differences in permeability between infected and non-infected cells in the case of wheat rust (8).

A comparison of the portions of aerobic CO_2 production which are sensitive to NaF in healthy and diseased tissue reveals approximately equal rates and clearly indicates, in conjunction with the above considerations, that the respiratory pathway responsible for the higher rates in diseased tissue during sporulation is distinct from that operating in healthy tissue.

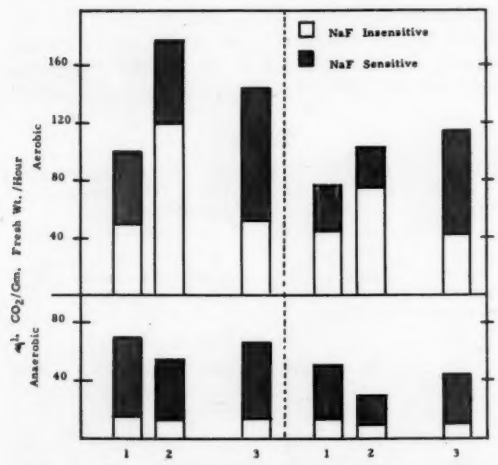

FIG. 1. The effect of NaF on CO_2 production aerobically and anaerobically in two separate plantings of safflower. 1. Healthy hypocotyls. 2. Rust-infected hypocotyls. 3. Healthy hypocotyls treated with 10^{-6} M DNP. See text for details.

C_6/C_1 RATIOS IN HEALTHY AND SPORULATING INFECTED HYPOCOTYLS: In view of the fact that safflower contains high amounts of lipid materials, there are two alternative mechanisms to account for both the decreased sensitivity to NaF and the low fermentation rates of diseased tissue: preferential lipid oxidation to two carbon fragments or operation of the hexose monophosphate shunt. A determination of the relative contributions of the first and sixth carbons of glucose to aerobic CO_2 production demonstrates that the direct oxidation pathway is of major importance in rusted tissue but of lesser magnitude in healthy tissue (table III). If glucose is metabolized by the Embden-Meyerhof glycolytic pathway, carbons 1 and 6 are equivalent and the C_6 to C_1 ratio of isotopic CO_2 produced should be equal to unity. The existence of a direct oxidative pathway reduces this ratio to values below one, as pointed out by Beevers and Gibbs (4). Healthy tissue has a ratio of 0.63 indicating some activity of the shunt, while the extremely low value of 0.18 for sporulating rusted tissue indicates much greater glucose breakdown via this pathway.

Beevers and Gibbs (4) have summarized the major assumptions in this type of experiment, including minimal assimilation of transformation of glucose into other substrates that might release CO_2.

THE NATURE OF DNP-STIMULATED RESPIRATION OF HEALTHY TISSUE. For several reasons indicated in the discussion, it is of interest to determine whether the host tissue is capable of the high level of activity of the hexose monophosphate shunt which obtains in diseased tissue. It is possible to increase the respiration of non-infected tissue to rates comparable to those of infected hypocotyls of the same age (5). A comparison of tissue treated with DNP and of normal tissue indicates that the respiration increased in this way is mediated by the Embden-Meyerhof system.

TABLE III

C_6/C_1 RATIOS IN HEALTHY AND SPORULATING RUST-INFECTED SAFFLOWER HYPOCOTYL

TISSUE	ISOTOPE	TIME (HRS.)	TOTAL MICROMOLES CO_2	CPM/MICROMOLE CO_2 PRODUCED	% RECOVERY	C_6/C_1
Healthy	1-C^{14}	5.25	24.1	187	0.60	0.63
	1-C^{14}		23.4	189	0.58	
	6-C^{14}		23.0	109	0.34	
	6-C^{14}		23.7	126	0.40	
Diseased	1-C^{14}	3.00	25.9	425	1.46	0.18
	1-C^{14}		25.4	393	1.34	
	6-C^{14}		27.5	73	0.26	
	6-C^{14}		26.1	74	0.26	
Healthy + 10^{-6} M DNP	1-C^{14}	4.00	22.8	150	0.46	0.73
	1-C^{14}		22.3	139	0.40	
	6-C^{14}		23.2	103	0.32	
	6-C^{14}		21.9	108	0.32	

In figure 1, the rates of anaerobic CO_2 production and its inhibition by NaF in healthy tissue treated with 10^{-5} M DNP are similar to that of untreated healthy tissue. Aerobically, the rates in treated tissue are much higher but the amount of respiration *insensitive* to NaF is the same with and without DNP, indicating that all of the extra CO_2 production induced by DNP is inhibited by NaF. This is in marked contrast to the respiration of infected tissue, where the percentage inhibition of respiration by NaF is lower than for untreated healthy tissue. In some experiments where only O_2 consumption was measured, there was nearly complete inhibition of respiration of DNP-stimulated tissue by NaF. Measurement of the C_6/C_1 ratio supports the experiments with inhibitor since the ratio of DNP-treated tissue is closer to unity (table III) than is untreated tissue.

Discussion

Although the evidence is indirect, three distinct types of experiments indicate that nearly all of the additional respiratory activity associated with sporulating infections of rust on safflower hypocotyls is mediated by the hexose monophosphate shunt. A comparison of our isotope data with those of Beevers and Gibbs (4) shows that the shunt is participating in metabolism to a much higher extent as a consequence of rust sporulation than is the case in a variety of normal plant tissues. Whether this is unique for rust infection or is characteristic of other plant infections is under current study, but the experiments of Sempio (summarized by Allen (1) in fig 1) demonstrate a similar change in the Pasteur effect induced by powdery mildew on wheat. If an RQ only of unity is assumed, ratios of anaerobic to aerobic CO_2 as low as 0.15 are obtained. As in our experiments, these low ratios with powdery mildew occur in the later stages of infection when sporulation would be heavy.

Although mycelial development of rust of safflower is accompanied by higher rates of respiration, it is not accompanied by a lowering of the ratio of anaerobic to aerobic CO_2 below 0.33 (5) nor has it been possible to demonstrate clearly a greater shunt activity at this stage of development of the parasite. Failure to do so, however, may be a result of the uninvaded tissue of the host masking the respiratory contribution of the relatively small quantities of mycelium and infected cells. Allen and Goddard (3) originally demonstrated that the host was responsible for the higher rates in wheat infected with powdery mildew and recent papers on mildew of barley (7) and bean rust (10) support this view. As a working hypothesis, it was postulated (5) that in safflower rust the respiration of the host is increased during mycelial development because of the metabolic activities arising in response to infection, rather than "uncoupling" toxins. During mycelial development, safflower hypocotyls are stimulated to rapid additional growth when compared to uninfected hypocotyls and the higher rates at that time probably result primarily from an increase in turnover of the adenylic system of the host as a consequence of growth. It is of interest that Gibbs and Beevers (6) have shown that glycolytic breakdown is predominant in immature and growing tissue. The present experiments show that the host has an NaF-sensitive pathway, presumably the Embden-Meyerhof pathway, capable of attaining the rates found during mycelial development.

The pronounced difference between normal and diseased sporulating tissue in the magnitude of the direct oxidative pathway suggests that the parasite may be responsible for the higher rates in the later stages of development of rust infected safflower. Although the parasite might induce large changes in the pathways of host tissue respiration, the results with DNP indicate the probability that higher rates would be mediated via glycolysis. It is more feasible that as the mycelium of the parasite, respiring through a hexose monophosphate shunt, increases in amount its contribution to respiration eventually surpasses the contribution of host tissue. Techniques other than those employed in previous studies must be used in order to establish the relative roles of host and parasite in respiration.

Such a situation might permit the application of the principle of selective toxicity for chemical control of disease through the development of anti-sporulants acting on the shunt, provided the shunt is of minor magnitude or can be bypassed in host metabolism. The data of Beevers and Gibbs show that considerable variation among species and with age of tissue is to be expected in this respect. In addition to possible importance for control, the recognition of the direct oxidative pathway as a major metabolic route in safflower rust delineates some of the physiological aspects of plant infections and may lead to a better understanding of the nature of obligate parasitism.

Summary

A comparison of healthy and rust-infected hypocotyls of safflower demonstrated a marked inhibition of the Pasteur effect during sporulation of the parasite, resulting in ratios of anaerobic to aerobic CO_2 production below a value of 0.33. Associated with the decrease in anaerobic CO_2 production in infected tissue is a decrease in the effectiveness of NaF as an inhibitor of respiration. There is also a considerable increase in the relative contribution of the first carbon of glucose to respiratory CO_2 in sporulating infections as indicated by a decline in the C_6/C_1 ratios measured with isotopes. The data suggest that during sporulation of rust on safflower hypocotyl nearly all of the greater respiratory activity arising from infection is mediated by the hexose monophosphate shunt, while in uninfected tissue the Embden-Meyerhof pathway is the major pathway.

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MOISTURE TRANSFER BETWEEN PLANTS THROUGH INTERTWINED ROOT SYSTEMS [1,2]

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The transfer of materials from roots to the soil has received considerable attention (1, 3, 4, 7, 8, 15), yet the transfer of materials from one root to another has received little study, despite the fact that the roots of most plants intimately grow together in soil. That material transfer between plants can occur was demonsrated by the early work of Breazeale (3) and Breazeale and Crider (4) in which wheat seedlings survived on water obtained from the roots of other plants. Recently, Kuntz and Riker (10) have shown that fungus spores, dyes, and poisons can be transported between oak trees through root grafts.

A great deal remains to be learned on the general problem of material transfer between roots. Little is known of the species capable of material transfer, the kinds of materials that move between root systems, the rate and magnitude of their transfer, the mechanisms of transfer, the environmental conditions under which transfer occurs, or the physiological importance of transferred material to the plants involved. Other questions of importance are whether transfer is primarily intra- or interspecific, whether movement of materials is polar or nonpolar when two species are involved, or whether transfer is a general phenomenon occurring in all vegetational situations or an isolated one occurring only under specific circumstances.

To make an experimental approach to the above problems a technique has been developed and several experiments on moisture transfer between plants have been completed. The purpose of these exploratory experiments was to determine whether significant moisture transfer would occur between relatively large tomato plants and to evaluate the physiological importance of the transferred water. An effort was made to assess the effect of certain environmental conditions, e.g., soil, on the success of transfer.

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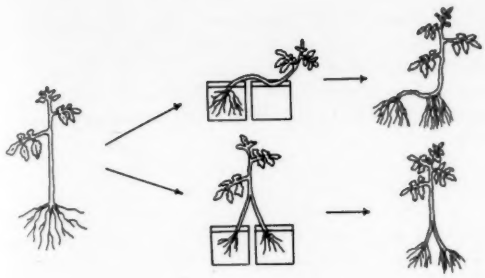
It is hoped that this will be the first of a series of experiments on material transfer between plants and that this and future work ultimately will allow an evaluation of the physiological and ecological importance of material transfer on the behavior of species and the mechanics of vegetation.

Methods and Results

General Technique: The experimental technique, based on the development of two root systems on a single plant, allows an evaluation of the transfer of material from one plant to another through adjacent intertwined root systems.

Double root systems can be developed on tomato seedlings in 7 to 10 weeks after germination in at least 3 ways. First, the exposed root system of a young seedling can be separated into two halves each of which is planted in a separate container thus allowing two discrete root systems to develop. This method was used in experiment one. Second, the stem of a seedling can be bent over, pegged to the soil surface of an adjacent container, and covered with soil and sphagnum moss at the point of contact. In about two weeks, during which both containers are kept moist, a second root system will develop (fig. 1). This method was used in all experiments but the first. In the third method, the root system and lower stem of a robust seedling are split and the halves are planted in separate containers. The exposed split, covered with moist sphagnum, soon calluses over and two discrete root systems develop (fig. 1). The latter two methods allow more flexibility in use than the first.

To permit evaluation of material transfer between plants, a donor plant with two root systems is planted with an experimental or recipient plant. One root system of the donor plant, the donor absorbing or DA system, is planted singly in a container of soil, while the other, the donor transferring or DT root system, is planted in another container along with the roots of the experimental or E plant. Following a period of adjustment during which intimate contact is established between the DT and E root systems, materials



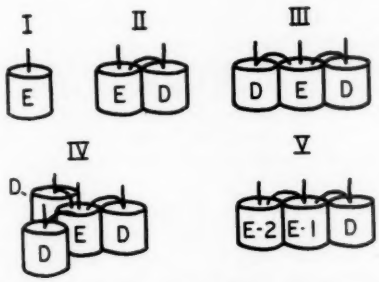


FIG. 1 (*above*). Two methods used to develop double root systems on young tomato seedlings. Above, bending and pegging; below, splitting.

FIG. 2 (*below*). Experimental groups. I represents an experimental plant with no donor; II, III, IV represent experimental plants whose roots are entwined with donor transferring roots of one, two, and three associated donor plants, respectively; V E-2 represents an experimental plant connected with a donor plant through an intervening double rooted experimental plant, V E-1.

such as water or radioactive ions are added to the container holding the DA system and withheld from the container holding the DT and E systems. After an appropriate interval of time, the E plant is checked directly or indirectly for the presence of the material. A positive result indicates that the material was absorbed by the donor plant through its DA system and transferred through its DT system to the experimental plant.

A number of variations on this basic technique are possible. More than one DT system may be planted with one experimental plant (fig 2, III, IV), or the experimental plant may be one or more times removed from the donor plant (fig 2, V). Additionally, the effect of soil on transfer may be tested by varying the media in the containers; for example, sand, loam, or clay may be used. Also, to eliminate the disturbing factor of absorption of ions by soil, the DA system may be grown in a beaker of aerated water to which test ions can be added. Environmental conditions influencing transfer, such as temperature, aeration, or light, also may be controlled.

EXPERIMENTAL WORK

During 1954 and 1955 four experiments were conducted in the Emory University greenhouse to determine the occurrence, rate, and importance of water transfer between intertwined root systems. In all experiments Marglobe wilt resistant tomatoes were used.

EXPERIMENT 1: This experiment was designed as a preliminary study of the transmission of water through adjacent root systems.

In July of 1954, fourteen pairs of plants consisting of an experimental plant and a single donor plant were prepared (fig 2, II). For each pair, two waxed one-pint ice cream containers were stapled together. The DA root system of the six-inch donor plant was planted in one container while in the other the DT system was planted along with the roots of a 6-inch experimental plant. A mixture of two-thirds loamy sand plus 1/6 manure plus 1/6 peat moss was used as a soil mixture in both containers. The paired containers were separated from one another by a layer of aluminum foil and were placed in dry flats.

After a two-week adjustment period, two groups of seven pairs each were randomly selected and subjected to drought from August 15 to 22. In the first group, 1 to 7, neither plant of a pair received any water during the drought period. In this case the "donor" plant was in reality a second experimental plant. In the second group, 8 to 14, the DA system of the donor plant was watered regularly while the containers holding the DT and E systems received no water.

During the drought period the experimental plants in both groups and the "donor" plants in the 1–7 group were scored for turgidity in accordance with the turgidity scale presented in table I. When the majority of the plants in the 1–7 group reached the severely wilted stage, on August 22, all plants were watered and recovery of turgidity was checked at hourly intervals for 6 hours.

RESULTS OF EXP'T 1: The 1–7 E group wilted most rapidly and on August 22, 86 % of the plants had reached the severely wilted stage (table II). The 1–7 "D" group followed the same trend, but showed a lesser degree of wilting with 29 % in the severely wilted category and 71 % in the wilted stage. The slightly better performance of the 1–7 "D" group might be because the "D" plants had access to two containers of soil while the E plants were restricted to one container.

TABLE I

TURGOR CATEGORIES DURING EXPERIMENTS 1, 2, AND 3

CATEGORY	TURGOR CONDITION OF PLANT
0	Fully turgid. No obvious sign of wilting.
1	Turgid. All but the lowermost leaves turgid.
2	Moderately wilted. Only the uppermost three or less leaves turgid.
3	Wilted. All leaves flaccid but not tightly curled.
4	Severely wilted. All leaves wilted and tightly curled, stem shriveled.

The 8–14 E group showed less tendency to wilt than either of the above groups, on the last day of drought only 14 % were severely wilted while 29 % were fully turgid (table II).

Recovery occurred in an inverse fashion to that of wilting. The 8–14 E group recovered most rapidly. After 4.5 hours, 86 % and 14 % were in the fully turgid and turgid categories, respectively. Groups 1–7 E and 1–7 D, after 5.5 hours, had only 29 % fully turgid, with 14 % severely wilted and 57 % in intermediate categories (table II).

These results indicate that roots of a plant not receiving water directly, but associated with roots of another plant receiving water through another root system, are able to obtain water from that plant in a sufficient quantity to delay wilting. Consequently, when water is again made directly available, recovery is speeded up. The ability of some plants in the 8–14 E group to maintain maximum turgor during an extensive drought period indicates that substantial quantities of moisture must be transferred in some instances.

Experiment 2: This experiment was designed to test the effect on moisture transfer of more than one donor plant and to determine the possibility of serial transfer through an intermediate plant. The effect of transferred water on the post-drought condition of experimental plants was also investigated.

Five different groups of plants were used in this experiment. Group I E consisted of experimental plants with no associated donor plants (fig 2, I). Groups II E and III E experimental plants had one and two associated donor plants, respectively (fig 2, II, III). Group V E–1 experimental plants, with double roots, had one system associated with a DT root system and the other associated with the roots of another experimental plant (fig 2, V E-1). Group V E-2 experimental plants were once removed from donor plants and could receive water only through intervening V E-1 plants (fig 2, V E-2). Group I E was replicated ten times, II E eight times, III E nine times, and V E-1 and V E-2 had seven replications each.

Waxed ice cream containers and the same soil used in experiment one were used here.

A drought period lasting from August 31 to September 8 was begun after the root systems in the various groups had become well established. Water was withheld from all E plants while it was added regularly to all D plants through their DA root systems. A regular watering schedule was reinstituted for all plants on September 8.

During the drought period and the first twenty-four hours of the recovery period all E plants were scored in accordance with the turgidity scale in table I. Approximately ten days after general watering was reinstituted, all experimental plants were examined and the number of dead, completely defoliated, and relatively normal plants was recorded along with the number of dead leaves on the normal plants. Growth and interdevelopment of adjacent root systems was also noted.

Results of Exp't 2: Group I E, with no donor root systems, showed the first signs of severe wilting and with the exception of one plant wilted relatively rapidly thereafter (table III). Group V E-2, whose connection with a donor plant was through an intervening experimental plant, did not show severe wilting as early as I E, but once severe wilting began this group wilted rapidly. The three remaining groups of experimental plants, all with one or more associated DT root systems, retained turgidity longer than the I E and V E-2 groups. Group V E-1 wilted more slowly than II E, perhaps because V E-1 plants ob-

Table II

Experiment 1. Percentage of Plants in Various Turgor Categories during Drought and Recovery Periods

Group	Turgor Category	Drought (August 15 to 22)						Recovery (hrs after watering)					
		15	17	18	19	20	22	0.5	1.5	2.5	3.5	4.5	5.5
II E * (1–7)	0	100	..	..	..	...	..	..	..	..	..	14	29
	1	...	..	..	..	...	..	..	..	..	..	..	14
	2	...	14	43	14	...	..	..	..	..	29	43	43
	3	...	86	57	86	100	14	29	29	71	57	29	..
	4	...	..	..	..	...	86	71	71	29	14	14	14
II D (1–7)	0	100	..	..	..	...	..	..	..	..	14	29	29
	1	...	..	43	..	...	..	..	..	14	14	14	29
	2	...	43	14	43	43	..	..	..	29	29	29	14
	3	...	57	43	57	57	71	71	71	43	29	14	14
	4	...	..	..	..	...	29	29	29	14	14	14	14
II E** (8–14)	0	100	14	14	14	29	29	29	29	29	43	86	86
	1	...	14	14	14	14	..	..	..	14	29	14	14
	2	...	43	43	43	43	14	14	14	43	14	..	..
	3	...	29	29	29	14	43	43	57	14	14	..	..
	4	...	..	..	..	...	14	14	..	..	..	..	..

* Donor plants in replications 1–7 received no water during drought period.
** Donor plants in replications 8–14 received water throughout experiment.

TABLE III

EXPERIMENT 2. PERCENTAGE OF PLANTS IN VARIOUS TURGOR CATEGORIES DURING THE DROUGHT PERIOD, SEPTEMBER 1 TO SEPTEMBER 8

GROUP	TURGOR CATEGORY	DATE							
		1	2	3	4	5	6	7	8
I E (10) *	0	100	10	10	10	...	...	...	...
	1	...	30	..	..	...	...	...	...
	2	...	30	10	..	10	10	...	...
	3	...	30	60	30	...	...	...	...
	4	...	..	20	60	90	90	100	100
V E-2 (7) *	0	100	29	14	..	...	...	...	...
	1	...	43	29	..	...	...	...	...
	2	...	14	29	14	...	...	...	...
	3	...	14	29	71	...	...	...	...
	4	...	..	..	14	100	100	100	100
V E-1 (7) *	0	100	43	14	14	...	...	...	...
	1	...	43	71	14	...	...	...	...
	2	...	14	..	57	...	...	...	...
	3	...	..	14	14	57	57	...	...
	4	...	..	..	..	43	43	100	100
II E (8) *	0	100	13	13	..	...	...	...	...
	1	...	25	13	13	...	...	...	...
	2	...	38	13	13	...	...	...	...
	3	...	25	63	63	25	25	...	...
	4	...	..	..	13	75	75	100	100
III E (9) *	0	100	56	33	33	11	11	11	11
	1	...	..	11	..	...	...	...	...
	2	...	22	22	..	11	11	...	...
	3	...	22	33	56	22	33	11	11
	4	...	..	..	11	56	45	78	78

* Number of replications.

tained some water from V E-2 containers early in the drought period. This reaction was similar to that of the 1-7 "D" plants of experiment one. Group III E, with two donors, wilted most slowly with one plant failing to lose turgidity throughout the drought period.

Why two donor plants were more effective than one in supplying an experimental plant with moisture is puzzling. Perhaps two DT systems associated with the roots of an experimental plant increase the surface through which water exchange can take place. Another possibility is that an increased volume of soil water is indirectly available to the experimental plant. If the latter is so, a single donor with a double volume of soil might be as effective as two donors with single volumes of soil.

Recovery, based only on plants capable of recovery with dead, completely defoliated, or turgid plants excepted, proceeded in a fashion inverse to that of wilting (table IV). Group III E recovered most rapidly, 25 % reaching full turgidity within two hours, 50 % in 3 hours, and 100 % in 8 hours. Group V E-1 showed the next most rapid recovery closely followed by II E. Groups V E-2 and I E recovered most slowly. Although several plants of the I E group recovered more rapidly than plants in the V E-2 group, at the end of 24 hours all plants in the latter group had recovered full turgidity while only 60 % of those in the former group had achieved this degree of turgor.

Observations made approximately ten days after the drought treatment ended revealed striking differences between groups (table V). Four out of ten plants in the I E group were dead and a fifth was completely defoliated and only beginning to show new growth. In all other groups no dead plants were found; one defoliated plant was counted in the V E-2 group, but it was observed that the transferring root system of its E-1 partner was poorly developed. Apparently direct or indirect contact with the root system of a donor plant allowed the transmission of enough water to avert death in all instances and to avert complete defoliation in all but one instance and the validity of this instance was questionable. It seems particularly significant that plants in contact with other plants in turn in contact with donors averted the disastrous effects that befell plants with no contact, direct or indirect, with donor plants.

Even relatively normal plants showed significant aftereffects of contact with donor plants during the drought period. Experimental plants associated with two donor systems had only 0.1 dead leaves per plant, while plants in contact with one donor plant, II E and V E-1, had an average of 0.9 and 1.7 dead leaves, respectively. V E-2 plants with indirect donor contact had 2.4 dead leaves, while I E plants with no contact had an average of 3.8 dead leaves (table V).

TABLE IV

EXPERIMENT 2. PERCENTAGE OF PLANTS IN VARIOUS TURGOR CATEGORIES DURING RECOVERY FROM WILTING, SEPT. 8, 9 A.M., TO SEPT. 9, 9 A.M.

GROUP	TURGOR CATEGORY	HRS AFTER WATERING									
		0	1	2	3	4	5	6	8	10	24
I E (5) *	0	...	...	...	...	..	..	20	40	60	60
	1	...	...	...	20	20	40	20	20	...	...
	2	...	...	...	...	40	20	20	...	...	...
	3	...	...	20	20	..	..	..	...	...	40
	4	100	100	80	60	40	40	40	40	40	...
V E-2 (6) *	0	...	...	...	...	..	..	..	17	50	100
	1	...	...	...	...	..	..	33	50	33	...
	2	...	...	...	...	17	33	33	17	17	...
	3	...	...	...	...	17	33	..	17	...	...
	4	100	100	100	100	67	33	33	...	...	...
V E-1 (7) *	0	...	...	...	14	43	71	71	86	100	100
	1	...	...	...	29	29	29	29	14	...	...
	2	...	...	14	43	29	..	..	...	...	...
	3	...	...	43	29	..	..	..	...	...	...
	4	100	100	43	...	..	..	..	...	...	...
II E (8) *	0	...	...	...	13	25	63	75	88	88	100
	1	...	...	...	13	25	25	13	...	13	...
	2	...	...	13	25	25	..	..	...	...	...
	3	...	12	25	25	13	..	13	13	...	...
	4	100	88	63	25	13	13	..	...	...	...
III E (8) *,**	0	...	...	25	50	63	75	88	100	100	100
	1	...	13	13	25	25	25	13	...	...	...
	2	...	25	13	13	13	..	..	...	...	...
	3	13	13	38	13	..	..	..	...	...	...
	4	88	50	13	...	..	..	..	...	...	...

Data based on plants with at least a few leaves capable of recovery.

* Number of plants capable of recovery.

** Does not include one plant that failed to wilt.

TABLE V

EXPERIMENT 2. CONDITION OF EXPERIMENTAL PLANTS TEN DAYS AFTER DROUGHT TREATMENT

GROUP	PLANTS PER GROUP	DEAD PLANTS	LIVING PLANTS		
			PLANTS WITH ALL LEAVES DEAD, NEW GROWTH ONLY	PLANTS WITH FEW DEAD LEAVES, NORMAL GROWTH	AV. NO. DEAD LEAVES PER NORMAL PLANT
I E	10	4	1	5	3.8
V E-2	7	0	1*	6	2.4**
V E-1	7	0	0	7	1.7†
II E	8	0	0	8	0.9
III E	9	0	0	9	0.1

* Transferring root system poorly developed.
** No record of dead leaves on 2 plants.
† No record of dead leaves on 1 plant.

The results of this experiment indicate that plants in direct or indirect contact with donor plants can obtain sufficient water from adjacent root systems to delay the on-set of wilting and, consequently, to speed-up recovery when water is again made directly available. Perhaps more important, this experiment indicates that for a period of time experimental plants in direct or indirect contact with donor plants can obtain quantities of water sufficient to prevent serious defoliation and death that occurs when such contact does not exist.

EXPERIMENT 3: Moisture transfer in previous experiments occurred between root systems growing in a loamy sand, peat, and manure mixture. This experiment was designed to determine the effect of another soil medium, sand, on the transfer of moisture.

A random block experimental design was employed in which each block contained three experimental plants: one with no donors, one with a single donor, and one with two donors (fig 2, I, II, III). Each block was replicated five times.

Double root systems were developed and groups were set up as in experiment two, however relatively pure medium textured sand was used as the soil medium. During the period of establishment the plants were watered with a dilute solution of Vigoro.

The drought period for the experimental plants lasted from the 10th to the 28th of September when watering of all plants was resumed. During the period of drought and recovery the turgor condition of the experimental plants was scored in accordance with the turgidity scale shown in table I.

At the conclusion of the experiment, observations were made on the number of living and dead experimental plants and the number of dead leaves per living plant. Also, an examination of associated E and DT root systems was made to determine their development, the extent of their intergrowth, and the existence of possible root grafts. This examination was facilitated by allowing the plants to wilt slightly, cutting the connection between the DT and DA systems, and allowing the DT portion to absorb a solution of eosin Y which, in turn, stained the DT system.

RESULTS OF EXP'T 3: The results, presented in table VI, are similar to those of earlier experiments. Group III E, with two donor systems, wilted most slowly and recovered most rapidly, while I E plants, with no donor system, wilted rapidly and recovered slowly. Group II E, with one donor, was intermediate in response.

Four out of five I E plants died as a result of drought, while no deaths were recorded in the other groups. The one surviving I E plant suffered greater leaf damage than the III E plants, while leaf damage to the II E group was intermediate.

The similarity between the results of this experiment using sand as a soil medium and those of previous experiments utilizing loam indicates that moisture transfer can take place under a range of soil conditions and that it is not always dependent on large amounts of colloidal matter in the soil.

Eosin uptake in most groups was considerable and examination of associated DT and E roots indicated that they were well developed and thoroughly intergrown. However, no root grafts were detected. At some points of contact between fine DT and E roots eosin *appeared* to be spreading from the DT roots over the surface of the colorless E roots.

EXPERIMENT 4: In all previous work, waxed ice cream containers with no provision for bottom drainage were used and it was thought possible that an accumulation of free water in the bottom of the DA containers might have influenced moisture transfer.

TABLE VI

EXPERIMENT 3. PERCENTAGE OF PLANTS IN VARIOUS TURGOR CATEGORIES DURING DROUGHT AND RECOVERY PERIODS

GROUP	TURGOR CATEGORY	DROUGHT (SEPTEMBER 10 TO 28)					RECOVERY* (HRS AFTER WATERING)			
		10	16	20	24	28	4	10	24	48
I E**	0	100	20	...	...	...	...	...	...	100
	1	...	..	...	...	...	...	...	100	...
	2	...	..	...	...	...	...	...	...	...
	3	...	20	40	...	...	...	100	...	...
	4	...	60	60	100	100	100	...	...	...
II E	0	100	40	...	...	...	...	...	100	100
	1	...	..	...	...	...	...	40	...	...
	2	...	40	20	...	...	...	60	...	...
	3	...	20	40	...	...	...	...	...	...
	4	...	..	40	100	100	100	...	...	...
III E	0	100	80	100	...	...	60	100	100	100
	1	...	20	...	20	40	40	...	...	...
	2	...	..	...	40	...	...	...	...	...
	3	...	..	...	20	60	...	...	...	...
	4	...	..	...	20	...	...	...	...	...

* All plants watered regularly beginning September 28, 8 A.M.
** Recovery data based on one living plant.

TABLE VII

EXPERIMENT 4. PERCENTAGE OF PLANTS IN VARIOUS TURGOR CATEGORIES DURING DROUGHT AND RECOVERY PERIODS

GROUP	TURGOR CATEGORY	DROUGHT (AUGUST 6 TO 12)							RECOVERY (HRS AFTER WATERING)			
		6	7	8	9	10	11	12	1	2	3	4
I E	1	50	25	...	50	...	...	...	...	...	...	25
	2	50	75	100	50	25	...	...	...	...	25	...
	3	..	..	...	...	25	25	...	...	25	...	...
	4	..	..	...	...	50	75	100	100	75	75	75
II E	1	25	50	...	100	...	...	...	...	...	...	100
	2	75	50	100	...	75	25	...	...	75	100	...
	3	..	..	...	...	25	75	50	50	25	...	...
	4	..	..	...	...	...	...	50	50	...	...	...
III E	1	25	25	...	25	...	...	...	...	...	25	75
	2	75	75	100	75	75	25	...	25	100	75	25
	3	..	..	...	...	25	50	75	75	...	...	...
	4	..	..	...	...	...	25	25	...	...	...	...
IV E	1	25	..	...	25	...	...	...	...	75	100	100
	2	75	75	100	75	100	100	75	100	25	...	...
	3	..	25	...	...	...	...	25	...	...	...	...
	4	..	..	...	...	...	...	...	...	...	...	...

To check this possibility a fourth experiment was performed during August of 1955 in which all donor and experimental plants were grown in clay pots with bottom drainage.

Four blocks, each composed of four experimental plants with zero, one, two, and three associated donor plants (fig 2, I, II, III, IV), respectively, were set up. Four-inch standard clay pots were used as containers and a loamy sand, peat, and manure mixture was used as soil. To minimize leaf area differences between experimental plants, all large leaves but the four uppermost were removed several days prior to the inauguration of the drought treatment.

Drought treatment for the experimental plants began on August 5 and ended on August 12 when all plants were thoroughly watered. Throughout the drought and recovery period the plants were scored for turgidity; however, the turgidity scale of table I did not portray accurately the various turgidity conditions. Perhaps this was because these plants, averaging sixteen inches in height, were taller and older than the experimental plants used in earlier work, and because many lower leaves had been removed.

Wilting seemed to proceed evenly over the whole plant and was scored in the following manner: 1) Fully turgid; 2) Turgid, terminal leaflets drooping; 3) Wilted, rachis arched and leaflets limp; and 4) Severely wilted, rachis arched, the angle between the petiole and the stem increased from the turgid angle, leaves rolled (particularly the uppermost) and the top of the stem drooping.

At the conclusion of the experiment, root development in several groups with intermingled E and DT systems was examined with a binocular dissecting scope and the observations were recorded.

RESULTS OF EXP'T 4: Results are presented in table VII. Plants in groups I E wilted most rapidly, and at the end of the drought period all were severely wilted. Groups II E had 50 % in the severely wilted stage at the end of the drought period, while III E and IV E had 25 and 0 %, respectively, in this stage. As in previous experiments, an increasing number of donor plants increasingly delayed wilting.

Recovery, based on all experimental plants, was quickest in IV E followed in order by III E, II E, and I E (table VII).

These results, based on donor plants in pots with adequate bottom drainage, are in agreement with those of previous experiments. This is indicative that standing water is not essential to adequate moisture transfer, and that moisture normally held in the soil can be absorbed and transferred by donor plants.

Microscopic examination indicated that E and DT root systems were well intergrown. In most instances roots tended to concentrate on the outer surface of the soil mass where many grew together in strands composed of several roots running parallel to the inner surfaces of the pot. Another experiment in which DT roots were marked with eosin indicated that the strands were composed of both E and DT roots. Some roots were found to be stuck together by a gelatinous substance which characterized their rhizospheres and which when pulled apart seemed to form a fibrous network. Apparently this same material caused soil particles to cling to roots. Root hairs were observed stretching between roots. No actual root grafts were seen, but the possibility of their existence could not be discounted.

DISCUSSION

It seems reasonably clear that the response of experimental plants was due to transferred water rather than to some other cause. Great differences in night and day temperatures might have resulted in condensation of some atmospheric moisture in E containers. If so, the importance of this moisture was probably

insignificant since E plants with no donors soon wilted while those with donors tended to remain turgid. Another explanation to be ruled out is that the results were due to differences in size of plants with larger donorless experimental plants wilting more rapidly than smaller experimental plants associated with donor plants. In three experiments, differences in size of experimental plants were more or less evenly distributed throughout all groups, while, in the fouth experiment, plants with no donors were almost always smaller.

Within the bounds of the experimental conditions, moisture was transferred between roots associated in either sand or loam, or through the roots of an intervening plant which itself had access only to transferred water. It was also established that standing water as a source for donor plants is not essential to successful transfer. Water held in potted soils with adequate drainage can be absorbed and transferred by donor plants in significant amounts. The amount of water received by an experimental plant was related to its proximity to the donor plant and to the number of donor plants. An increased number of donor plants improved the ability of E plants to maintain turgor. This might have been due to increased surface contact between DT and E roots resulting from the higher ratio of DT to E roots, or to more soil moisture being indirectly available to the experimental plant.

Water obtained from donor plants, directly or through the roots of an intervening plant, was physiologically significant. It was transferred in quantities sufficient to delay, or even prevent, the onset of wilting in relatively large plants. Consequently, the plants recovered full turgidity and maximum physiological activity more quickly when water was again directly made available. Transferred water also helped to avert or lessen reaction to drought resulting in loss of leaf area. In terms of recipient plants, transferred water not only increased survival, but also allowed them to achieve a greater degree of growth following drought than would have been possible without transferred water during the drought period.

The first step in moisture transfer is loss of water from the donor plant. Although several theories have been advanced to account for loss of water through roots under various environmental conditions—loss in response to tension gradients within continuous moisture films between the roots and soil particles (3), loss by exudation (2), and loss as vapor along a vapor pressure gradient between the root and the surrounding atmosphere (5, 6, 14)—the way in which water molecules moved out of the DT roots in these experiments is uncertain.

There is some indication that transfer occurred only at higher soil moisture tensions. Double rooted E-1 plants with one root system associated with turgid DT roots (expt 2) seemed at first to withdraw available moisture from associated E-2 containers and later to supply water to the same containers. This is in agreement with observations (1, 3) that double rooted plants with one root system in water or in continuously moistened soil withdrew all moisture from a moist soil down to the wilting percentage or built up the moisture content of dry soil to the wilting percentage. This suggests that donor plants withdrew water from E containers until some critical moisture or vapor tension was achieved after which, under appropriate conditions, water was supplied to the containers.

The pathways by which water molecules move from DT root systems to E root systems are obscure. However, at least four possibilities seem to be plausible: 1) from the DT system to soil particles to the root system of the E plant; 2) movement through direct cellular contact between closely appressed roots and root hairs of DT and E systems: 3) movement between adjacent roots, not in cellular contact, through "gelatinous" material that seemed to characterize their rhizospheres; and 4) movement through fine root grafts between DT and E systems.

A plant to soil to plant transfer of moisture was advanced by Breazeale (3) and Breazeale and Crider (4) to explain the survival of small wheat seedlings whose roots were entwined with those of turgid plants in dry soil. This view is partially supported by other evidence that indicates a plant with a portion of its root system in moist soil can lose water to a dry soil through another portion of its roots (1, 3, 4, 7, 15). Breazeale and Crider (1934) suggest that an accurate measurement of water lost in this way would reveal a thin zone of relatively moist soil adjacent to the root, however, it is generally thought that the moisture content of the dry soil is raised only to approximately the wilting percentage (9). Presumably some of the water absorbed by dry soil could be absorbed by the roots of another plant.

A morphological basis for moisture transfer between adjacent roots through closely appressed rootlets or root hairs or through a "gelatinous" matrix extending between them was observed (expts 3 and 4). Examinations of E and eosin-marked DT root systems indicated that roots of both were abundant and intertwined and that many points of direct contact existed between them. The opportunity for transfer by either of the above methods seemed particularly good in instances where young roots of both systems were growing together in relatively long strands composed of 4 or 5 roots plus a common "gelatinous" matrix. These strands might have been caused by the confining nature of the pots, or possibly water deficient E roots may have grown parallel to turgid DT roots in response to the moisture available there. In many places several roots were stuck together by a "gelatinous" substance concentrated in their rhizospheres near the root apices. In single roots, this material apparently caused soil particles to cling to roots causing a soil sheath effect similar to that described by Magistad and Breazeale (13).

Perhaps, sloughed off cells of the root cap or cortex are broken down by micro-organisms to form a heterogeneous "gelatinous" matrix which in turn forms a

connection between the root and surrounding soil particles. Water molecules and ions might migrate through this substance toward the root in response to gradients established by activity of the root. Under certain conditions, transfers between roots might occur when their "gelatinous" zones come into contact and a gradient is established between them.

The only evidence for transfer of moisture between roots through cellular or "gelatinous" contact was a single observation made under difficult conditions in which eosin seemed to spread from a DT root along the surface of an appressed E root (expt 3).

No actual root grafts were observed, but accurate observation was extremely difficult because of the thorough intergrowth of the E and DT root systems. It is not unreasonable to suspect grafts may have occurred between succulent tissues of adjacent roots growing under the confined conditions in the containers. Root grafting is known to occur under natural conditions, particularly in woody plants (10, 11, 12). Recently Kuntz and Riker (10) demonstrated the movement of dyes, fungus spores, and poisons between oak trees through root grafts.

The only evidence for grafting in these experiments is of an indirect nature. Several experimental plants were able to maintain almost complete turgor throughout their respective drought periods suggesting a more efficient means of moisture transfer than plant to soil to plant or contact transfer.

Root grafts as a method of transfer seem most unlikely in some instances. Since grafts between distantly related species are unlikely, moisture transfers between tomato and wheat (4) and sunflower and tomato (pilot experiment) indicate that some other means of transfer was operative in these cases. When considering the problem of material transfer in its widest context, however, grafts between species cannot be automatically dismissed since they are known to occur (11).

The evidence at hand indicates that the four methods of transfer may work individually or, perhaps, together in various combinations depending on the circumstances. The determination of the relative importance of each method will have to await further experimentation. Techniques involving the use of radioactive isotopes, large molecules like 2,4-D, biological agents such as plant viruses, and interspecific transfers show promise of resolving these problems and of helping to assay the importance of material transfer in nature.

Summary

The general problem of material transfer between plants is briefly discussed and an experimental method for its evaluation is explained. Data from four experiments on the transfer of moisture between tomato plants are reported.

Moisture was transferred between plants whose roots were associated in sand or loam, or through the roots of an intervening plant which itself had access only to transferred water. It moved in quantities sufficient to delay, or even prevent, the onset of wilting in relatively large plants.

The mechanism of transfer is discussed and it is suggested that transfer occurs only after some critical soil moisture tension is achieved. Several pathways of water movement between roots are suggested.

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TRANSLOCATABLE PLANT GROWTH INHIBITORS PRODUCED BY PENICILLIUM THOMII AND ARACHNIOTUS TRISPORUS[1,2]

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In 1954 a screening program was initiated at Purdue University which has as one of its objectives the detection of microbiologically-produced compounds which are able to influence the growth of higher plants. It is the purpose of this paper to report the detection of translocatable plant growth inhibitors produced by *Penicillium thomii* Maire and *Arachniotus trisporus* Hotson.

MATERIALS AND METHODS

The fungus cultures used in these studies were maintained as soil stocks. Conidial suspensions from seven-day-old potato-dextrose-agar slant cultures prepared from soil stocks were used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of corn steep–cerelose medium (Staley's corn steep liquor, 40.0 gm; cerelose, 40.0 gm; $CaCO_3$, 3.5 gm; $NaNO_3$, 3.0 gm; K_2HPO_4, 0.5 gm; $MgSO_4$, 0.25 gm; de-ionized H_2O, 1000 ml). Two drops of Dow Corning Antifoam AF were added to each flask prior to autoclaving. After inoculation the flasks were placed on a reciprocating shaker (99 to 100 cpm with 3-inch strokes) at 27 to 28° C for 7 days. At the end of this time the mycelial growth in each flask was removed by filtering through Whatman No. 1 filter paper and discarded. The culture filtrates were adjusted to pH 5.0 and one drop of Tween 80 per 100 ml (approx.) of filtrate was added. The undiluted filtrates were frozen until needed. Filtrates from approximately 700 fungi were collected in this manner. Approximately 350 of these were unidentified soil isolates obtained by routine plating-out procedures and 350 were identified as to genus and/or species. In addition, 500 "actinomycete" culture filtrates obtained from cultures routinely isolated from soil were obtained from the Eli Lilly Company of Indianapolis, Indiana. With two exceptions these filtrates showed no antibiotic activity toward a wide spectrum of microorganisms. The methods used in obtaining these culture filtrates were similar to those described above for the fungus cultures. The shake-flask medium, adjusted to pH 7.0, was as follows: Bacto-peptone, 5.0 gm; glucose, 10.0 gm; molasses (Brer Rabbit Green Label) 20 ml; $FeSO_4 \cdot 7H_2O$, 0.01 gm; and distilled water, 1000 ml. The inoculated flasks were placed on a reciprocating shaker (114 cpm with 2-inch strokes) for 5 days at 30° C. As with the fungus cultures the growth in each flask was removed by filtration and discarded. The culture filtrates were treated as described above and frozen until needed.

Bean (*Phaseolus vulgaris* L., var. Black Valentine) and corn (*Zea Mays* L., the single cross WF9 × 38-11) seedlings were used as test plants and grown under ordinary greenhouse conditions. The crude filtrates were ordinarily used directly with no dilution.

The bean seedlings, grown in vermiculite without added nutrients in individual 4-inch pots, were treated when the primary leaves had expanded to about one-half of their normal size and before elongation of the epicotyl had begun. The culture filtrates were applied by placing 40 to 50 droplets (ca. 0.3 to 0.4 ml) on only one of the primary leaves on each of two alternate days. At the end of 7 to 10 days the plants were examined for any effects on the treated leaf and on the subsequent growth. Usually, 2 to 4 plants were used for each treatment.

Corn seedlings (6 plants per pot) were grown in soil in 6-inch pots. When the seedlings were 6 to 8 cm in height they were treated by merely filling the whorls with the culture filtrates on each of two alternate days. These were allowed to grow for 10 to 12 days at which time their heights were measured and effects of the treatment were noted. In all cases, water and the uninoculated culture medium (without carbon source) served as controls. Twelve to 18 plants were used for each treatment.

RESULTS

Although some 1200 culture filtrates were tested for their effects on higher plants it is the purpose here to present in detail only the results obtained when plants of corn and bean were treated with the culture filtrates of *P. thomii* and *A. trisporus*. When the primary leaves of bean plants were treated with the culture filtrates of these two organisms the treated leaves became chlorotic, slightly necrotic, and were smaller than the untreated leaves. Most interesting, however, was the effect on the compound leaves which subsequently developed. The treated plants did not develop symmetrical trifoliolate leaves but were inhibited more or less selectively on the side of the treated primary leaf to give an asymmetrical leaf. Figure 1 illustrates the effect of culture filtrates of *A. trisporus* (1-, 2-, and 3-week shake-flask incubation) on the first trifoliate leaves when one of the primary leaves was treated. Similar results were obtained when plants were treated with culture filtrates of *P. thomii*. The effect extended, in many cases, to the third and fourth compound leaves, although with diminishing magnitude. Actually, leaf expansion on both sides of the compound leaves was inhibited, but the inhibition was greatest on the side of the treated primary leaf.

The culture filtrates could be reduced to dryness at 55 to 60° C in a vacuum or forced air oven without loss of activity. Beans were treated at 0.1 and 10 times the normal culture filtrate concentration (fig 2).

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Little or no effect could be noted at 0.1 times the normal concentration but was striking at 10 times the normal concentration. Inhibition of the elongation of the epicotyl was more apparent at the higher concentration. None of the results described above were obtained by treating the plants with the uninoculated culture medium.

Corn plants treated with culture filtrates of *P. thomii* and *A. trisporus* were chlorotic, somewhat necrotic and much smaller than the controls. The average heights of 24 plants treated with filtrates of *P. thomii*, *A. trisporus*, uninoculated medium, and water were 33.4, 27.8, 40.0 and 45.5 cm, respectively.

In addition to *P. thomii* and *A. trisporus* culture filtrates from the following fungi were found to produce similar effects on corn and beans: unidentified soil isolates designated only as 55-6, 55-115, 55-116, unidentified species of Arachniotus and of Gymnoascus, and recently, *Penicillium oxalicum*, a fungus which has been reported to produce oxalic acid (1). Oxalic acid at concentrations ranging from 10,000 to 0.1 ppm was used in treating bean plants and failed to produce the effects described above.

Because of their relatively close taxonomic relationship to *P. thomii* and *A. trisporus* a number of identified penicillia and aspergilli were included in the 700 fungus filtrates tested. In summary, 41 spp. of Aspergillus and 37 spp. of Penicillium failed to produce the inhibitors described above. Furthermore, the inhibitor could not be detected in culture filtrates of *Gibberella fujikuroi*, the organism which produces gibberellin and related compounds. These culture filtrates were, however, very active in stimulating the growth of corn and bean plants.

In addition to Black Valentine bean the translocatable inhibitor produced by *P. thomii* was found to produce similar effects on seedlings of Bountiful bean, soybean, and tomato. No activity was apparent in one test on seedlings of radish, muskmelon, and cucumber.

Attempts to obtain the translocatable inhibitor produced by *P. thomii* and *A. trisporus* on shake-flask media other than the corn steep–cerelose medium have failed. No activity could be detected, using bean as a test organism, on media of potato-dextrose broth, Raulin-Thom, Richard's, or Czapek-Dox even with incubation periods of two weeks. Growth of the fungi was poor on the Czapek-Dox medium, but good growth was obtained on the other media tested. The effects of the treatment on bean plants appeared to increase when the filtrates were obtained from corn steep–cerelose shake-flask cultures incubated for longer than one week (2 to 3 weeks).

The culture filtrates of *P. thomii* and *A. trisporus* were examined for antibiotic activity using the paper-disc method of Vincent and Vincent (9). No antibiotic activity was detected using *Ustilago maydis*, *Corynebacterium flaccumfaciens* (Gram-positive) and *Bacterium stewartii* (Gram-negative) as test organisms. However, if the culture filtrates were concentrated to 10 times their normal concentration small and faint antibiotic zones could be observed on plates of *C. flaccumfaciens*. The antibiotic produced by *P. thomii* was found to be soluble in ethanol and insoluble in benzene, chloroform and ether.

Using Black Valentine bean as an assay organism the following information has been obtained concerning the inhibitors produced by *P. thomii* and *A. tri-*

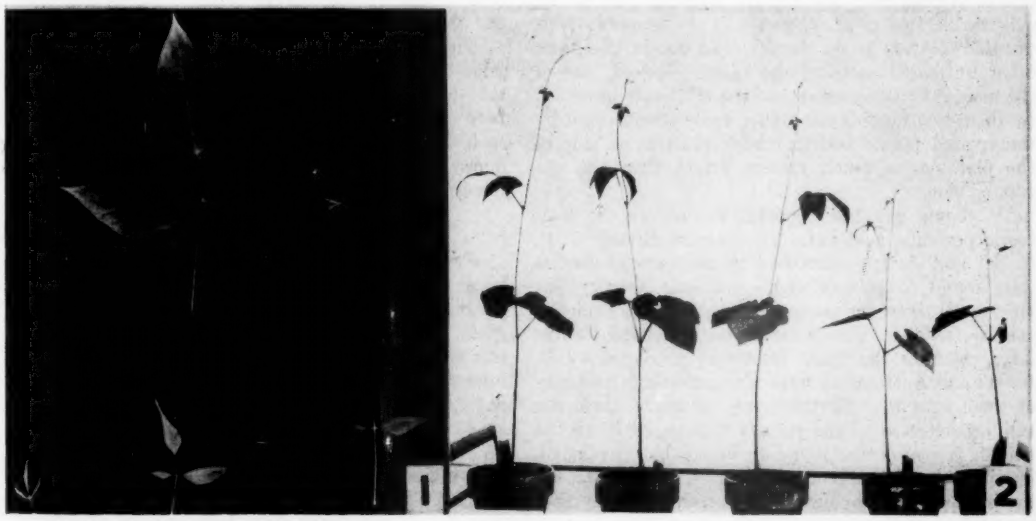

FIG. 1. First trifoliate leaves of bean plants which have been treated on one of the primary leaves with water (*above*) and culture filtrates of *Arachniotus trisporus* (*below*). From right to left (*below*) cultures incubated for 1, 2, and 3 weeks.

FIG. 2. Bean plants treated on one of the primary leaves with uninoculated medium, water, 0.1, 1, and 10× the normal culture filtrate concentration of *Penicillium thomii* (*left to right*). Shake-flask cultures incubated for 1 week.

sporus: the active compounds were stable to autoclaving at pH 5, 7, and 9 but not at pH 2, dialyzable through cellophane membranes, and not adsorbed on activated charcoal. They were soluble in methanol and ethanol but insoluble in benzene, chloroform, ethyl acetate and ethyl ether (pH 2, 7, 10). In addition, the inhibitor produced by *P. thomii* was found to be insoluble in carbon tetrachloride, n-amyl alcohol, petroleum ether and n-butanol. Whereas the inhibitor produced by *A. trisporus* was found to be insoluble in acetone and iso-propanol, the inhibitor produced by *P. thomii* is apparently at least slightly soluble in both of these solvents. Both inhibitors could be precipitated from ethanol by the addition of ethyl acetate.

Neither of the inhibitors produced by these fungi were adsorbed on the cation exchange resins IR-120 or IRC-50, but were adsorbed by the anion exchange resins IRA-400 (OH form) or Dowex 1-X 8. The active compounds were readily eluted from a column of IRA-400 with 0.75 % NaCl.

Discussion

The production by microorganisms of substances deleterious to the growth of higher plants is not unique. Culture filtrates of plant pathogenic microorganisms have been demonstrated on many occasions to produce compounds which adversely affect plant growth (5, 6, 8). This is especially true in the case of those pathogens which induce wilting in the host plants (2, 3, 7, 10). In most cases, however, the production by plant pathogenic organisms of substances deleterious to the growth of higher plants has been demonstrated by immersing the stems or roots of plants directly into the culture filtrates. Using the culture filtrates of *P. thomii* and *A. trisporus* it is possible to treat leaves directly and obtain effects on other untreated parts of the plant. Indeed, one of the most interesting aspects of the inhibitors produced by these two fungi is the ability to be translocated by the treated plants and to inhibit the treated side of the plants to a much greater extent than the untreated side.

P. thomii has been reported to produce the antibiotic penicillic acid (4). The culture filtrates of *P. thomii* and *A. trisporus* used in the present studies were found to possess slight antibiotic activity but only at 10 times the normal concentration. Penicillic acid is freely soluble in ether, benzene, and chloroform, whereas the plant inhibitors produced by *P. thomii* and *A. trisporus* were not extractable with any of these solvents. Furthermore, the slight antibiotic activity detected in the culture filtrates of *P. thomii* and *A. trisporus* was found to be soluble in ethanol, but insoluble in benzene, chloroform, and ether. On the basis of the above evidence it seems unlikely that the plant growth inhibitor produced by these fungi is related to penicillic acid.

The recent discovery that *P. oxalicum* produces a plant growth inhibitor similar to those produced by *P. thomii* and *A. trisporus* suggested that perhaps oxalic acid was involved. Since the culture medium contained an excess of $CaCO_3$, the inhibitor was not extractable with ether at pH 2, and oxalic acid in a wide range of concentrations failed to elicit the response when applied to plants, this possibility was ruled out.

Certain of the evidence on hand would indicate that the inhibitors produced by *P. thomii* and *A. trisporus* are related or identical compounds: 1) no differences have been observed in their effects on treated plants, 2) they are both heat stable, dialyzable, and not adsorbed on charcoal, 3) their solubility properties in methanol, ethanol, benzene, chloroform, ethyl acetate and ethyl ether are similar and 4) both are adsorbed by and can be eluted from anion exchange resins. That differences may exist between the two inhibitors is indicated by their solubilities in acetone and iso-propanol. When acetone or iso-propanol were added in excess to culture filtrates of *A. trisporus* the activity was found only in the precipitate which was formed. On the other hand, when similar treatments were made to culture filtrates of *P. thomii* both the soluble fraction and the precipitate possessed activity, despite repeated washing of the precipitate. The inhibitor produced by *P. thomii* appears to be at least slightly soluble in both of these solvents.

Some information is now available concerning the nature of the inhibitors produced by these fungi, but their identity is not known. The active compounds are small anions as indicated by dialysis and adsorption and elution from anion exchange resins. Although they were not extracted with ether at low pH values the possibility that they are organic acids must not be excluded, since not all organic acids are soluble in ether. Attempts are being made to purify and identify the active compounds.

Finally, it might be pointed out that although some 500 "actinomycete" culture filtrates were examined for the activity described above no filtrate has been observed to produce this effect. Whether or not such translocatable plant growth inhibitors are produced by this large group of organisms may be determined by the results of further screening.

Summary

Penicillium thomii and *Arachniotus trisporus* have been found to produce translocatable plant growth inhibitors when cultured on corn steep-cerelose medium. When one of the primary leaves of bean plants was treated with the culture filtrates of these organisms the compound leaves which develop later were not symmetrical but were inhibited more or less selectively on the side of the treated leaf. Stem elongation of bean plants and the growth of corn plants were also inhibited by treatment with these culture filtrates. The inhibitors were found to be heat stable at pH 5, 7, and 9 but were destroyed by autoclaving at pH 2, dialyzable, and not adsorbed on charcoal. They were soluble in methanol and ethanol but insoluble in benzene, chloroform, ethyl acetate and ethyl ether. Whereas the inhibitor produced by *A.*

trisporus was insoluble in acetone and iso-propanol, the inhibitor produced by *P. thomii* was apparently at least slightly soluble in both of these solvents. The inhibitors could be adsorbed by the anion exchange resins IRA-400 and Dowex 1-X 8 and eluted from the columns with 0.75 % NaCl.

The author is indebted to Professors K. B. Raper and M. P. Backus, University of Wisconsin, and Professor A. J. Ullstrup, Purdue University, for generously supplying the identified cultures used in these studies, and to the Eli Lilly Company, Indianapolis, Indiana, for the "actinomycete" culture filtrates.

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BRIEF PAPERS

THE EFFECT OF BIURET ON PROTEIN SYNTHESIS IN PLANTS[1,2]

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The foliar application of urea as a nitrogen source has now become an important agricultural practice. It has been reported, however, that application of commercial urea preparations to citrus (5) and to pineapple (6) results in leaf injury. The injury (a progressive, irreversible yellowing from the tip of the leaf) is not, however, attributable to urea itself, but is caused instead by small quantities of biuret which are present in many samples of commercial urea (3, 5, 6). Little is known concerning the mode of action of biuret on plant growth and metabolism. This communication reports results of exploratory studies on the effect of biuret on the metabolism of various nitrogenous substances (especially urea) in plants.

We have found that a convenient plant for the assay of biuret injury is *Xanthium pensylvanicum*. When young Xanthium plants, grown under the conditions described by Bonner and Thurlow (1), are treated with a solution of 0.5 % urea and 0.03 % biuret, marked leaf yellowing is evident five days later. Continued treatment with the urea-biuret mixture at five-day intervals results in a yellowing from the leaf tips progressively downward in a manner very similar to that observed in citrus. Neither urea alone nor biuret alone elicits this response within five days. Plants treated with biuret alone, however, begin to show yellowed leaves after 15 to 20 days.

The fact that biuret produces yellowing of leaves much more rapidly in the presence of urea than in its absence suggests that biuret affects either the absorption or the initial metabolism of urea. The results of both Hinsvark et al (4) and Webster et al (8) have suggested that urea is metabolized in leaves, at least in part, by hydrolysis to carbon dioxide and ammonia. Experiments, however, demonstrated that both the absorption of urea-C^{14} by Xanthium leaves, and its hydrolysis, either by extracts of Xanthium leaves or by purified jack bean urease, are inhibited only slightly (10 to 15 %) by the highest attainable concentrations of biuret. In addition, we have found biuret to be without effect on the ability of cell-free extracts of Xanthium leaves to oxidize any of several amino acids, to decarboxylate glutamate, or to hydrolyze arginine.

In contrast to this lack of effect on the metabolism of urea, biuret elicits a marked decrease in the protein content of leaves (fig 1). It is noteworthy that biuret produces greater decreases in protein level in the presence of urea than in its absence. This effect is in agreement with both our observations and those of Jones (5) that greater yellowing of leaves occurs in the presence of a mixture of urea and biuret than in the presence of biuret alone.

As leaf proteins are undoubtedly undergoing constant breakdown and resynthesis, the decrease in protein level produced by biuret could be due either to an inhibition of protein synthesis or to a promotion of protein decomposition. In order to learn whether biuret increases the rate of protein decomposition, Xanthium plants, including their proteins, were labeled by photosynthesis in $C^{14}O_2$. The plants, containing radioactive protein, were then treated with biuret or a mixture of biuret and urea, and the loss of radioactivity from the total leaf protein measured daily, and compared with the loss from untreated plants. This was measured by comminution of three treated and three untreated plants in 0.01 M KCl, removal of cellular debris by centrifugation at 500 × g for 10 minutes, and precipitation of the remaining protein with 12 % trichloroacetic acid. The protein was washed and assayed for radioactivity as described previously (7). No significant differences in the release of radioactivity could be detected, even though the protein level in the leaves treated with the biuret-

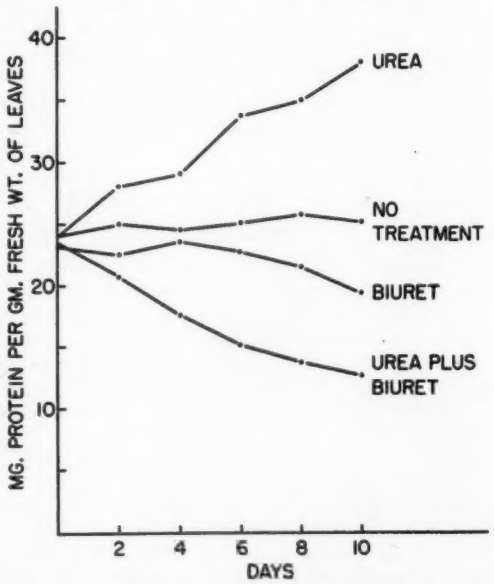

FIG. 1. Effect of urea and biuret on the protein levels of Xanthium leaves. Protein was determined by the method of Gornall et al (2).

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[2] Supported in part by the Polychemicals Department, E. I. duPont de Nemours and Co., Inc.
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TABLE I

EFFECTS OF BIURET AND A MIXTURE OF BIURET AND UREA ON THE INCORPORATION OF AMINO ACIDS INTO PROTEIN *

AMINO ACID	MICROMOLES AMINO ACID INCORPORATED/GM PROTEIN × HR		
	NO ADDITION	PLUS BIURET	PLUS BIURET AND UREA
Alanine-C^{14}	0.53	0.47	0.29
Glutamate-C^{14}	0.85	0.76	0.57
Leucine-C^{14}	0.18	0.16	0.11

* Amino acid incorporation by intact leaves, attached to the plant, was measured as described previously (7) following application of 10 micromoles (1,000,000 cpm) to the leaf surface. Leaves were treated with biuret or biuret plus urea five days before application of the amino acid.

urea mixture decreased markedly. These results suggest that biuret does not promote the rate of leaf protein decomposition in any striking manner.

Conversely, biuret inhibits the incorporation of C^{14}-alanine, C^{14}-glutamate, and C^{14}-leucine into the leaf proteins of intact Xanthium plants (table I). As is the case with protein level (fig 1), the greatest inhibition of amino acid incorporation into protein is produced by a mixture of biuret and urea. It would seem, therefore, that the decrease in leaf protein elicited by biuret may be due to an inhibition of protein synthesis. The exact manner in which this inhibition occurs is not yet clear. It could be due to: (a) an interference with amino acid production; (b) a direct inhibition of protein synthesis; or (c) a secondary effect evoked by the action of biuret on a completely unsuspected site. Likewise, the observation that a mixture of biuret and urea is a more effective inhibitor than biuret alone is susceptible to several interpretations. The fact remains, however, that the mechanism of leaf damage by biuret is probably not a simple case of biuret affecting some obvious phase of urea metabolism. Instead, the effect is apparently either on protein synthesis or on some equally basic process of metabolism that directly affects protein synthesis.

SUMMARY

Biuret, which elicits marked leaf injury in urea-treated Xanthium plants, has no significant effect on the following metabolic processes: absorption of urea by Xanthium leaves, hydrolysis of urea, oxidation of amino acids, decarboxylation of glutamate, hydrolysis of arginine, and breakdown of leaf protein. In contrast, biuret produces a sharp decrease in total leaf protein, and inhibits the incorporation of amino acids into leaf proteins. It is concluded that biuret, either directly or indirectly, inhibits protein synthesis.

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THE EFFECT OF PROLONGED SHADING ON THE LIGHT SATURATION CURVES OF APPARENT PHOTOSYNTHESIS IN SUN PLANTS[1,2,3]

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Comparative light saturation curves of apparent photosynthesis of plants whose natural environment is full sun and of plants which naturally grow in deep shade are given by Lundegårdh (5), Rabinowitch (6), and Böhning and Burnside (3). In studying these curves it becomes apparent that leaves of the sun plants usually have a higher compensation point and also become light saturated at a higher light intensity than those of the shade plants. These differences have been used as one of the means for classifying plants into the physiological categories of sun and shade types.

[1] Revised manuscript received October 18, 1956.

[2] Parts of this paper were taken from a thesis presented by the senior author in partial fulfillment of the requirements for the Master of Science degree.

[3] Paper from the Department of Botany and Plant Pathology, The Ohio State University. Number 592.

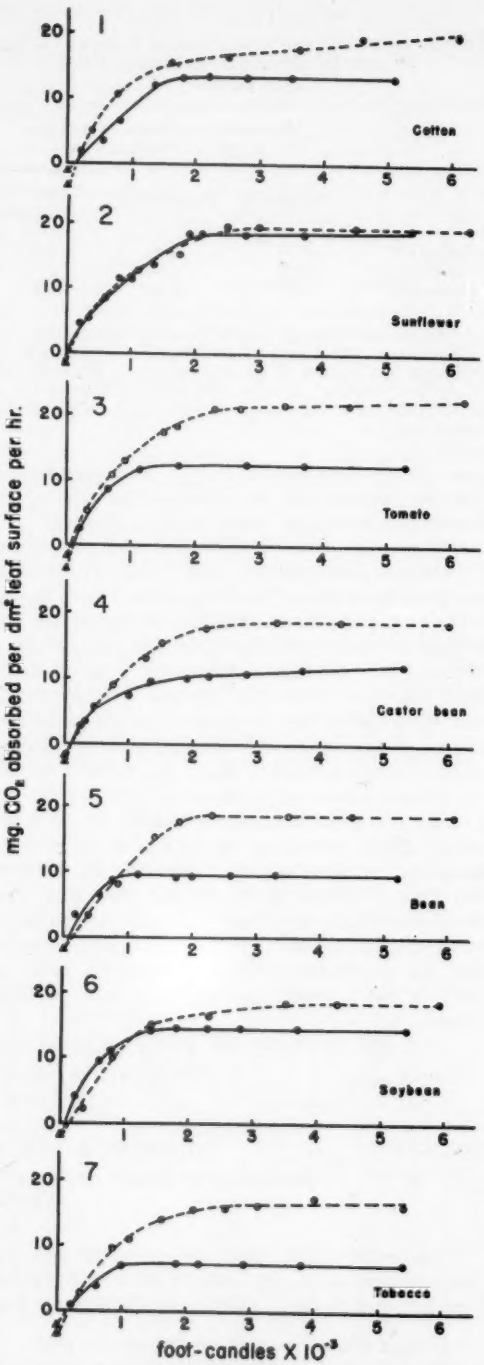
However, several authors have questioned this practice and have presented arguments against it. Thus Harder (4) demonstrated the rapid reversibility from sun to shade plants in Fontinalis, depending upon the light intensities to which they were exposed before and during the measurements of photosynthesis. Blackman and Wilson (1) found that "the net assimilation rate during the season of active growth is linearly related to the logarithm of the light intensity" in both sun and shade plants.

In the present investigation plants having leaves possessing the typical light curves of sun plants when growing in the full sunlight conditions of their natural environment (3) were grown under light intensities of 100 to 700 ft-c i.e., in deep shade. The species used were bean (*Phaseolus vulgaris* L. var. Black Valentine), tomato (*Lycopersicum esculentum* Mill. var. Marglobe), sunflower (*Helianthus annuus* L.), cotton (*Gossypium hirsutum* L. var. Coker 100), tobacco (*Nicotiana Tabacum* L. var. White Burley), soybean (*Glycine Max* (L.) Merr.), and castor bean (*Ricinus communis* L.). All developed from potted seeds in the greenhouse.

The experimental apparatus and methods used in making the photosynthetic measurements have been described previously (3) and only the main points will be reviewed briefly at this time. Measurements were made on single attached leaves inserted in a Plexiglas leaf chamber. An air stream of constant CO_2 concentration, provided by a compressed air tank, was passed over the leaf and the reduction in CO_2 concentration due to photosynthesis was measured by means of a Liston-Becker infra-red gas analyzer, model 15. Illumination was provided by six General Electric reflector flood lights mounted above a water bath. Photosynthesis was measured at light intensities ranging from 0 to 5500 ft-c. The initial CO_2 concentration supplied by the tanks of compressed air in the sun plant experiments was 288 ± 10 ppm. That in the shade plant experiments was 320 ppm. During the course of the measurements the mean CO_2 concentration in the leaf chamber was 270 ± 30 ppm for the sun-grown and 290 ± 10 ppm for the shade-grown plants.

Light saturation curves from these experiments are shown in figures 1 to 7. The sun leaf curves in these graphs apear elsewhere (3) and have been reproduced here for purposes of comparison.

The light curves of leaves of sun plants which grew under conditions of deep shade are strikingly similar to those of "true" shade plants. In all cases except sunflower and castor bean the saturation light intensity was at least 1000 ft-c lower in the shade-grown plants than in the sun-grown plants. The lowering of the compensation point ranged from 0 to 100 ft-c in all cases except in tomato, where it increased. Thus it has been shown that the saturation and compensation points of leaves of sun plants seem to depend upon the environmental light condition during their growth period. A plant whose normal habitat is in bright light can be caused to yield



FIGS. 1 to 7. Effects of light intensity on the rate of apparent photosynthesis in leaves of sun (broken line) and shade-grown (solid line) plants. ▲ represents dark respiration.

a light saturation curve strikingly similar to that of a plant whose normal habitat is in deep shade. Yet one would not wish to classify this plant as a "shade" type. It becomes increasingly evident that the classification of plants into sun and shade types solely on the basis of the light saturation curves of their leaves is questionable. The physiological classifications "sun" and "shade" plants are convenient, however, and perhaps should not be discarded. Accordingly, the authors feel in agreement with the views expressed by Blackman and Wilson (2) and Shirley (7), that plants should be arranged into sun and shade types on the basis of their ability to survive in a given light environment rather than on the basis of the light saturation curves of their leaves.

Summary

The rate of apparent photosynthesis in relation to light intensity has been measured in leaves of seven species. Measurements were performed on plants of each species which had developed in the greenhouse 1) in full sunlight and 2) at a light intensity ranging from 100 to 700 ft-c. All species had been determined previously as having the typical light curves of sun plants when growing under conditions similar to those of their natural habitat. With one exception leaves of the shade-grown plants were light saturated at a light intensity at least 1000 ft-c lower than those of the sun plants. The compensation points of the shade plants, with one exception, were lowered by 0 to 100 ft-c from those of the sun plants.

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CELL SPACE AND APPARENT FREE SPACE IN THE RED ALGA, PORPHYRA PERFORATA[1]

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Apparent free space has been defined as "that portion of a plant tissue into which substances in solution apparently move by free diffusion" (1). In order to estimate the intracellular concentrations of ions in the monostromatous red alga *Porphyra perforata* for permeability studies it was necessary first to get an approximation of the extra-cellular space. This was done in two ways: 1) a modification of the sucrose space method of Scott and Hayward (2), and 2) the phenol red technique commonly used in animal physiology. (Microscopic estimation indicates that about half of the thallus volume is occupied by cells and half by extra-cellular material; see Johansen (3), page 295.)

Discs of Porphyra were soaked for times up to 10 minutes in 0.6 M sucrose, or 0.1 % phenol red in sea water, removed, blotted twice with absorbent tissue and placed in a known volume (5 to 50 ml) of sea water. The amount of sucrose or phenol red which diffused out of the tissue into the sea water was determined; the results are expressed as percent of the concentration of the original external solutions (based on the fresh weight of the tissue).

Figure 1 indicates that the sucrose concentration in the discs reached a constant value within a minute

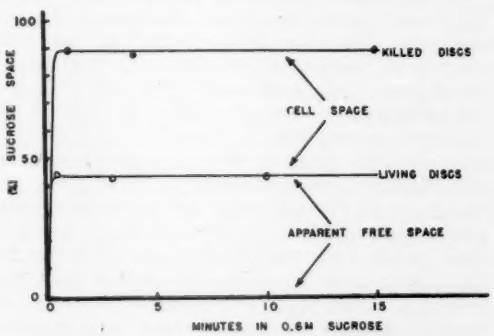

Fig. 1. Sucrose space of living and killed *Porphyra perforata* thalli as a function of time in 0.6 M sucrose solution. Units of sucrose space are percentages of the thallus volume (on a fresh weight basis) available to sucrose diffusion.

[1] Received October 23, 1956.
[2] Public Health Service Fellow of the National Heart Institute.

of exposure. The values for the apparent free space varied from 41.8 to 45.3 % with an average of 43.6 %. Phenol red gave the same value. For comparison sucrose and phenol red spaces were also determined for the local *Ulva Lactuca*. The values obtained were 43.4 % for the phenol red space and 33.7 % for the sucrose space. That the agreement between phenol red and sucrose spaces in Porphyra may be fortuitous is suggested by the lack of agreement of the two methods when applied to Ulva. Further indication of this comes from a comparison of the values obtained for killed discs of Porphyra. In this case the sucrose space was constant with time at 89 % while the phenol red space was about 250 %. This high value for phenol red space suggests a reversible adsorption of the dye on unknown components of the tissue. The dye is apparently a less reliable indicator of free space than is sucrose.

In the killed discs of Porphyra (killed by boiling or by cold 50 % ethanol) the sucrose space value was 89 %. Perhaps the remaining 11 % of the tissue volume represents a region of relatively low water content such as cell wall or lipid. The difference between the sucrose space values for living and killed tissue may represent the aqueous cell space, i.e., 45 % of the thallus volume.

Another problem met in approximating intracellular ionic concentrations in Porphyra was the adsorption of cations. Sodium was adsorbed to the extent of 40 meq/kg fresh wt as indicated by the amount of sodium remaining in the thalli after killing. Sodium in the living tissues was 60 to 80 meq/kg fresh wt. Thus more than half of the sodium in the tissue is adsorbed. Potassium varied from 180 to 220 meq/kg fresh wt in living plants while none was detectable in killed ones (corresponding to its low concentration in sea water). These values were obtained after dipping the tissue two minutes in 0.6 M sucrose, blotting twice with absorbent paper, weighing, and ashing with concentrated nitric acid; the extracts were made up to volume and the sodium and potassium were determined by means of the Beckman flame photometer. All the calcium in the tissue (8 to 10 millimoles/kg fresh wt) was apparently adsorbed, as determined by EDTA titration against murexide indicator at pH 12 (4) of living and killed plants after ashing.

Calculation on a cell space basis, after subtracting the adsorbed sodium, gives values of 400 to 490 meq/l cell space for potassium and 45 to 67 meq/l cell space for sodium, and a total of 445 to 557 meq/l cell space for both cations. If sodium and potassium were present as their chlorides the cells would be approximately isotonic with sea water.

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PLASTID PIGMENTS OF GAMETOPHYTES AND SPOROPHYTES OF MUSCI[1]

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It is common knowledge that both the gametophytes and the less conspicuous sporophytes of mosses are green. The sporophytes usually lose their green color at or before maturity. Bold (1) described the development and distribution of the green plastids in the sporophytes of certain mosses. He assumed that the green pigment was chlorophyll and that the presence of starch grains in the green plastids indicated that photosynthesis occurred in the sporophytes. Although Strain (2) and others have reported the presence of chlorophylls a, b and various carotenoids in moss gametophytes the author is not aware that any comparable data for moss sporophytes have been published. This paper is a report of the results of research directed toward answering two questions. Is the green pigment in the plastids of moss sporophytes chlorophyll? Are there qualitative differences between the pigments found in the haploid and diploid phases of mosses?

[1] Revised manuscript received November 20, 1956.

The mosses used in this study were *Entondon seductrix* Hedw., *Thuidium recognitum* Hedw., *Mnium sylvaticum* Linb., *Brachythecium acuminatum* Kindb., and *Ditrichum vaginans* Sulliv. These plants were collected over a period of two summers from their natural sites in northeastern Illinois at times when they were bearing green sporophytes. Subsequently the sporophytes were clipped from the gametophytes, with scissors, for extraction and analysis of the pigments of each generation. There was never any dearth of gametophytes but the amount of sporophytes was usually less than 2 gm, fresh weight.

The methods used in extraction, separation, and identification of the pigments were adapted from those outlined by Schertz (4), Strain (5), and Zscheile and Comar (6) as follows. In order to stop enzyme activity and facilitate extraction the plant material was killed by placing it in a test tube which was immersed in a beaker of boiling water for a few minutes. Extraction was carried out in a Waring

blender with acetone plus a pinch of calcium carbonate. The pigments were transferred to petroleum ether by partition in a separatory funnel. Saturated NaCl solution was used for diluting the acetone to aid the separation and prevent the loss of any xanthophylls. The petroleum ether fraction was washed about ten times with the salt solution to remove all traces of acetone.

Carotenes were separated from the other pigments at this point by decreasing the volume, under reduced pressure, of the petroleum ether extract and then pouring it on a 5-cm layer of powdered sucrose in a small Buechner funnel with a fritted disc plate. The sugar layer was prepared as a slurry in petroleum ether and compressed and rubbed smooth on top while still moist. Pure petroleum ether was added to the filter until all of the carotenes were removed with the filtrate. This filtrate was then passed through a magnesium oxide, silicious earth (1:1) adsorption column. The chromatogram was developed with petroleum ether. The bands of carotenes were eluted with petroleum ether and analyzed in a spectrophotometer.

Chlorophylls and xanthophylls were adsorbed in the upper centimeter of the sugar filter with the bulk of the xanthophylls being below the chlorophylls. The pigments were mechanically separated into two fractions one consisting of xanthophylls and the other containing chlorophylls plus some xanthophylls. The fraction of pure xanthophylls was eluted with ethyl ether. The sugar fraction containing a mixture of chlorophylls and some xanthophylls was eluted with methyl alcohol and these two classes of pigment were separated by partition as follows. Petroleum ether was added to the methyl alcohol solution of pigments and shaken. Then enough saturated salt solution was added to dilute the methyl alcohol to 80 %. After shaking and separation the methyl alcohol layer with xanthophylls was drained off. Extraction of the petroleum ether fraction, containing chlorophylls, with methanol, diluted to 80 % with salt solution, was repeated three times. Xanthophylls in the combined 80 % methanol fractions were transferred to ethyl ether by partition after more dilution with salt solution. The resulting ether solution of xanthophylls was combined with the one previously obtained, dried over anhydrous sodium sulfate, concentrated to a very small volume under reduced pressure, taken up in petroleum ether, and passed through a magnesium oxide, silicious earth adsorption column. The chromatogram was developed with a 2:1 (v/v) solution of petroleum and ethyl ether. Each band of xanthophyll was noted with respect to position and color and then eluted with ethanol for analysis in the spectrophotometer.

The petroleum ether solution of chlorophylls, separated previously, was washed several times with water to remove all traces of methanol, dried over anhydrous sodium sulfate, reduced in volume to about 10 ml, and added to a Tswett adsorption column composed of powdered sugar. The chromatogram was developed with a 4:1 solution of petroleum and ethyl ether. Each band of chlorophyll was eluted with ethyl ether for analysis in the spectrophotometer.

A Beckman, model DU, spectrophotometer was used to determine the absorption spectrum of each pigment. Individual carotenes and xanthophylls were identified by their relative positions on the respective adsorption columns and by their maximum light ab-

TABLE I

PLASTID PIGMENTS IN SPOROPHYTES AND GAMETOPHYTES OF MOSSES

MOSS TISSUE	CHLOROPHYLL		CAROTENES		XANTHOPHYLLS				
	a	b	α	β	NEOXANTHIN	VIOLAXANTHIN	ZEAXANTHIN	LUTEIN	CRYPTOXANTHIN
Entodon seductrix									
Gametophyte	x	x	x	x	x	x	x	x	x
Sporophyte	x	x	x	x	x	x	x	x	x
Ditrichum vaginans									
Gametophyte	x	x	x	x	x	x	x	x	–
Sporophyte	x	x	x	x	x	x	x	x	–
Brachythecium acuminatum									
Gametophyte	x	x	x	x	–	x	x	x	–
Sporophyte	x	x	x	x	–	x	x	x	–
Mnium sylvaticum									
Gametophyte	x	x	x	x	–	x	x	x	–
Sporophyte	x	x	x	x	–	x	x	x	–
Thuidium recognitum									
Gametophyte	x	x	x	x	–	x	x	x	–
Sporophyte	x	x	x	x	–	x	x	x	–

Presence of pigment is indicated by x.

sorption bands as reported by Strain (2, 5) and Kay and Phinney (3).

The results of this research are summarized in table I. From these data it is obvious that qualitatively there is little or no difference between the pigments in the gametophyte and sporophyte generations of the mosses considered in this investigation. Chlorophylls a and b, α and β carotenes, and the xanthophylls, lutein, vialoxanthin, and zeanthin, were consistently found in both generations. In addition neoxanthin was present in two genera and cryptoxanthin in one. These two xanthophylls as well as others may have been present in trace amounts in the other mosses since small amounts of unidentified xanthophylls were present in all of the moss samples. In chromatographing the xanthophylls a faint band was usually located at or near the top of the column. This band appeared to be a mixture in that after elution its absorption spectrum had no marked absorption peaks. In addition traces of unidentified xanthophylls often came through the adsorption column. These trace xanthophylls comprised a very small part of the total carotenols and were not further investigated. The small samples, especially of sporophytes, used in this investigation were a limiting factor in the identification of any carotenoids which may have been present in small amounts.

The data provide positive evidence that the green pigments of moss sporophytes are chlorophylls and that the green plastids in the sporophytes are in fact chloroplasts. This is further evidence in support of the idea that moss sporophytes are partially, if not entirely, independent of the gametophytes with respect to food.

With respect to the major plastid pigments in mosses the data in table I indicate that doubling the chromosome number per cell, going from haploid gametophytes to diploid sporophytes, does not materially alter the kinds of major pigments which the cell can synthesize. The situation may be different with regards to the amount of each pigment formed. Such a quantitative study was not undertaken since there are apparently many environmental factors which could alter the results.

Summary

The plastid pigments of the gametophytes and sporophytes of five mosses, *Entodon seductrix, Thuidium recognitum, Mnium sylvaticum, Brachythecium acuminatum,* and *Ditrichum vaginans,* were determined by conventional methods of differential solubility, column chromatography, and spectrophotometry.

Chlorophylls a and b, α and β carotene, lutein, violaxanthin, and zeaxanthin were found in both the sporophytes and gametophytes of all of the mosses. In addition neoxanthin was identified in both generations of *Ditrichum vaginans* and *Entondon seductrix*. The latter species also contained cryptoxanthin. The data indicate no qualitative differences in the major pigments of the haploid and diploid generations of a particular moss, and that the green plastids in moss sporophytes are chloroplasts.

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American Society of Plant Physiologists, *Annual Meeting, Storrs, Connecticut, August 1956:* The American Society of Plant Physiologists held its 31st Annual Meeting from August 26th to 30th, 1956 on the campus of the University of Connecticut in connection with the annual convention of the American Institute of Biological Sciences. This was the largest meeting our society had ever sponsored. A total of 205 papers was presented on our program, 181 being contributed papers and 24 comprising symposial discussions. Once again, there was completely satisfactory cooperation between our group, various sections of the Botanical Society of America and the American Society for Horticultural Sciences. Our society also participated in and aided the Golden Jubilee celebration of the Botanical Society of America.

The local committee, under the chairmanship of Professor Arthur E. Schwarting of the School of Pharmacy, performed an outstanding job of arranging facilities for the meetings. As in last year's meeting, all papers were held rigorously to the time allotted to them on the program. One innovation which proved popular with the delegates was that all papers were scheduled for fifteen minutes, including discussion time, and a coffee break was scheduled at 10:00 each morning. Contributed papers were limited to the morning sessions and only symposia were scheduled for the afternoon sessions.

Additional copies of the abstracts of the meetings, published as a supplement to volume 31 of the journal, were placed on sale near the meeting rooms. One hundred and forty-six (146) of these were sold. Additional copies are available from the Secretary at fifty cents (50¢) each. This practice of printing extra copies of the abstracts for sale at the meetings will be continued next year.

Many comments were heard about the high quality of most of the papers, the general improvement in the caliber of the work described, and the nature of the slides presented. Some concern was expressed over the continued increase in the number of papers presented at the annual meetings. If this trend continues, we shall face a serious situation in a few years. Meetings may have to be limited in one way or another, or the society may have to encourage regional meetings to supplant or at least partially replace the national meetings.

Executive Committee Meeting, *August 26th:* The Executive Committee of the society met in room 236 of the Pharmacy Building, University of Connecticut. The meeting was opened at 7:15 P.M. by President Harry A. Borthwick. Present were: H. A. Borthwick, A. S. Crafts, H. J. Dyer, A. W. Galston, D. R. Goddard, P. J. Kramer, J. L. Liverman, R. H. Muir, A. W. Naylor, and K. V. Thimann. Invited to present special reports were V. A. Greulach, L. Machlis, M. W. Parker, A. E. Schwarting and F. C. Steward.

Reading of the minutes of the past meeting was dispensed with, as the minutes had been published in the January issue of *Plant Physiology*. President Borthwick reported that the affairs of the society appear to be in good shape. Membership and subscriptions have increased somewhat; the financial condition appears to be strong; and the journal is constantly increasing in quality and in promptness. A replacement editor for Dr. Goddard has been found. A new crisis in personnel, which arose with the resignation of Dr. J. Fisher Stanfield, for the last 12 years Executive Secretary-Treasurer of the organization, has also been solved on a temporary basis by a satisfactory replacement. The Charles Reid Barnes Life Membership Award Committee, under the chairmanship of Hugh Gauch and including also O. Biddulph, R. H. Burris, P. J. Kramer and Jack Myers chose H. B. Vickery of the Connecticut Agricultural Experiment Station as the recipient for this year. The Stephen Hales Committee, consisting of Birgit Vennesland, chairman, L. R. Blinks and F. Skoog, chose Melvin Galvin of the University of California as the next recipient, citing him for his able charting of the path of carbon in photosynthesis. Dr. E. J. Kraus, former chairman of the Memorial Committee, asked to be relieved and Dr. A. E. Murneek was appointed to his place. As his first act, Dr. Murneek has prepared a statement concerning the recent death of Dr. W. W. Garner. K. V. Thimann and W. A. Andreae were appointed to the Editorial Board of *Plant Physiology*, P. J. Kramer was appointed to the Board of Trustees, and H. A. Borthwick retained his position as representative on the Editorial Board of the *American Journal of Botany*.

The secretary reported on the election of new officers of the society. Folke Skoog of the University of Wisconsin was chosen president; S. B. Hendricks of the U.S. Department of Agriculture, vice-president; A. W. Galston continues as secretary for another year; K. V. Thimann of Harvard University was elected a member of the Executive Committee, and R. B. Withrow of the Smithsonian Institution, Washington, D. C. and R. S. Bandurski of Michigan State University were elected members of the Editorial Board. Two hundred and forty-four (244) nomination ballots and five hundred and thirty-nine (539) election ballots were received. The latter represents approximately a 50% response of the membership to the balloting.

The large number of contributed papers, coupled with the three major afternoon symposia, necessitated the scheduling of four concurrent sessions on each of four mornings. The printing of the abstracts followed last year's plan with 3000 copies being ordered instead of the 2500 printed last year. The cost were as follows:

Printer	$ 975.00
Stamps	125.55
Mailing	10.50
	$1111.05

The cost, therefore, is about 34¢ each. The extras are to be placed on sale at the meetings at 50¢ a copy. A new feature of the abstract supplement is that each session is given a title and a table of events summarizing the sessions is included on the first page.

Vocational guidance literature continues to be popular. Approximately two requests per week for information come to the secretary's office.

The Executive Secretary-Treasurer presented a report summarizing the financial status of the organization as follows.

MEMBERSHIP:

	July 1, 1955	July 1, 1956
Life members	33	34
Corresponding members	19	19
Canadian	30	39
U. S.	949	1014
Puerto Rico, T. Hawaii & Alaska	26	28
Foreign members	101	110
	1158	1244
Less those included in other groups	5	5
	1153	1239

SUBSCRIPTIONS:

	Vol. 30	Vol. 31
U. S.	387	398
Foreign	845	890
	1232	1288

SUMMARY OF CASH ACCOUNTS:

Checking account, July 1, 1955	$15,537.27
Savings account, July 1, 1955	14,000.00
Cash balance, July 1, 1955	$29,537.27
Receipts, 1955–56	34,313.07
Total	$63,850.34
Expenditures, 1955–56	34,207.30
Cash balance, July 1, 1956	$29,643.04

Cash on hand, July 1, 1956

Checking account	$11,943.04
Reserve account	17,700.00
Total	$29,643.04

SUMMARY OF ENDOWMENT ACCOUNT:

(*Life Membership and General Endowment Fund*)

Balance, July 1, 1955	$ 660.74
Income, 1955–56	
Barnes Life Membership Awards (1955 & 1956)	250.00
Sales of back numbers of journal	2,454.10
Excess Barnes & Hales	61.56
Interest on investments	1,030.19
Two life membership purchases	200.00
Bonds called and matured	5,287.51
Total	$ 9,944.10
Expenditures, 1955–56	
Bond purchases	9,317.60
Balance, July 1, 1956	$ 626.50

Dr. Arthur E. Schwarting reported for the local committee and acknowledged the great help rendered by Dr. Byron Janes. The banquet, room arrangements, projection equipment and miscellaneous facilities have been satisfactorily provided. The committee expressed its appreciation to Dr. Schwarting and his helpers for the excellent arrangement which had been made.

M. W. Parker, our representative for the American Association for the Advancement of Science, read a letter from the AAAS requesting that our society contribute $25.00 per year to help council members attend the annual meetings. The Board agreed with the principle of aiding representatives to attend, but made no appropriation at the time.

Dr. F. C. Steward, our representative to the National Research Council, reported on the satisfactory resolution of the situation regarding the allocation of United States Public Health Service grants. Our society, along with other scientific groups, had expressed last year to the National Academy of Sciences and to its president Dr. Detlev Bronk, its concern over the use of political criteria of acceptability of awarding of such grants. Dr. Steward assures us that through Dr. Bronk's action on behalf of the various interested groups, this situation has now greatly improved. Dr. Steward also reported that a survey is desired by the American Physiological Society on the utilization of retired physiologists. It was moved and passed that our society express its interest in this project.

Dr. Goddard gave a report on the status of the journal, *Plant Physiology*, as of August 15, 1956.

Papers received (July 1955 to date)		151
Papers accepted	83	
Papers published *	58	
Papers for publication **	25	
Papers withdrawn	6	
Papers rejected	21	
Papers for revision	12	
Papers in process	29	
Papers for decision	8	
Papers being reviewed	19	
New papers	2	
* Papers published		115
Papers received before July 1955	57	
Papers received after July 1955	58	
** Papers for publication		26
Papers received before July 1955	1	
Papers received after July 1955	25	
Papers at press	20	
Papers on hand	6	

Dr. Goddard's overall view of the status of the journal was very optimistic. The quality of the articles is rising, there are fewer rejections, and we are operating on current manuscripts. It now takes about 6 months for a paper to be published. Twenty-five hundred and twenty-seven (2527) copies of the journal are now being circulated and we are still growing. The cost of the journal to the society is now $20,000 per year, of which $2500 is paid for half-time services of an editorial assistant.

With regard to the Executive Secretary-Treasurer's report, K. V. Thimann commented that the proceeds from the sale of life memberships and back issues of the journal go into the Endowment Fund rather than into the general operating funds for the journal. To change the undesirable situation in regard to the life memberships, a constitutional amendment would be required; the matter of the back issues is a long-standing procedure, but not constitutionally required. The Board of Trustees was requested to study this situation and report back to the Executive Committee on ways of ameliorating it.

P. J. Kramer, our representative to the AIBS, reported that the organization is growing and is vigorous, having now solved the problem of how to raise money for conferences and various research projects. It has a visiting lecturer's bureau, can help with mailings and the negotiations of group insurance, and will probably soon take over the *Quarterly Review of Biology*.

Dr. V. A. Greulach reported that his committee, including H. B. Tukey, C. H. Wadleigh, C. J. Lyon, T. C. Broyer, and H. M. Benedict had conducted a survey based largely on a questionnaire and was ready to report to the society on the status of plant physiologists in industry. This report was enthusiastically received and it was recommended that the report of the committee be published in *Plant Physiology*. The committee was also encouraged to prepare a new and enlarged plant physiology career booklet.

A committee under K. V. Thimann, including H. B. Vickery, B. S. Meyer, F. Skoog, A. S. Crafts, and D. R. Goddard reported on the new editor for *Plant Physiology*. Allan H. Brown of the University of Minnesota was chosen for this office and has accepted as of the January issue, 1958. Until that date, Dr. D. R. Goddard of the University of Pennsylvania will continue as editor. It was moved and passed that beginning on January 1, 1958, the editor receive an annual honorarium of $1000.00 for his services, exclusive of the expenses of publishing and editing the journal.

Dr. Borthwick reported with regret that because of illness, Dr. and Mrs. J. Fisher Stanfield have been forced to relinquish the duties of Executive Secretary-Treasurer. Dr. G. Ray Noggle of the C. F. Kettering Foundation, Yellow Springs, Ohio, has taken over these duties on a temporary basis. The Executive Committee unanimously voted that he be asked to continue in this office for a three year term. Dr. Noggle accepted this appointment and was voted a $500.00 per annum honorarium in addition to his expense account of $800.00 yearly for the running of his office. A committee, consisting of A. W. Naylor and A. W. Galston was empowered to draw up a statement expressing the society's appreciation to Dr. and Mrs. Stanfield for their long years of service, such statement to be incorporated into a scroll to be presented to them.

The meeting was adjourned at 11:30 P.M.

ANNUAL BUSINESS MEETING: More than 300 people attended the annual banquet and business meeting. The main business consisted of 1) a report to the membership on the Executive Committee Meeting and the status of the society, 2) presentation of the Charles Reid Barnes and Stephen Hales awards, 3) introduction of new officers and 4) a retiring presidential address by Harry A. Borthwick.

The following statement, prepared by A. W. Naylor and A. W. Galston at the behest of the Executive Committee was read by the secretary.

"The American Society of Plant Physiologists is today a vigorous scientific organization of more than 1200 members. Its present strength is largely the result of the devoted and selfless service of a relatively small group of loyal members. Among these, Dr. and Mrs. J. Fisher Stanfield have been outstanding contributors to the welfare of our group. On this, the occasion of Dr. Stanfield's retirement from the duties of the Executive Secretary-Treasurer's office, we wish to express to them our admiration for the magnitude and quality of the work done and our heartfelt gratitude for the spirit and devotion with which this has been performed. We hope and trust that in his retirement, he will continue to give us of his wisdom, a wisdom born of constant association with the society from its inception to the present time."

The president noted with regret the death of two of our members, W. W. Garner and H. B. Pulling.—Respectfully submitted, ARTHUR W. GALSTON, Secretary.

CURRENTS IN BIOCHEMICAL RESEARCH—David E. Green, editor. Interscience Publishers, Inc., New York; Interscience Publishers, Ltd., London. 697 pages. 1956. $10.00.

There is hardly a biologist, let alone biochemist, who would not eagerly look into a book that purports "to communicate to non-specialists an overall impression of the present status of the significant problems in each field, to point up the broad strategy of current research, and, finally, to speculate on the likely paths of future research." Many readers may not agree with editor David Green that "progress" during the ten years under survey has been "vastly greater than in any comparable period since the early beginnings of biochemistry." Yet, every decade, depending on mood, sees its image as golden or gaunt, and the extravagance will be easily forgiven. For it must be admitted that this has been a decade extraordinarily abundant in print, if not intellect, and to the harrassed researcher or perplexed academician the prospect of a *book* bringing reasonable light to the ten-year stacks of journals, reviews, and symposia, is most attractive. The lofty and exciting introduction leads the reader to suppose that the book has been composed after ample reflection, without haste, and without the usual frantic search for exhaustive references. A work so thoughtfully conceived should indeed whet the appetite of any scholar.

So much for claims, hopes, and wishes. The book

is hardly a scholar's dream, although it would probably be judged as good by the standards usually applied to scientific publications. The contributors are competent and accomplished. To question the details of their essays is beyond the reach of this reviewer. The selection of topics, on the other hand, is within critical reach and has proved mildly disagreeable. Plant physiologists will be surprised to learn that of the 27 biochemical currents covering "nearly every important field of biochemical research" one lonely current (photosynthesis) has its origins in botanical waters. Of the remainder, 3 are devoted to biosynthetic pathways, 9 to enzyme chemistry, 9 to physiological functions, 3 to protein and nucleic acid chemistry, one to biological continuity, one to embryology, one to theory, and *none* to cytochemistry or biochemical genetics. The list might easily have been reduced or expanded; circumstance more than design seems to have dictated the contents. The topics are unevenly treated, a situation we have come to expect for multiauthored books. There is a superbly conceived speculative piece of Lipmann; excellent reviews on photosynthesis (Bassham and Calvin), disease (De Witt Stetten, Jr.), electron transfer (Chance) and blood (Surgenor): and a host of other good essays. By contrast, there is an overly tendentious piece on adaptive enzyme formation (Spiegelman and Campbell), a complicated article on hormones (Pincus) which to the non-specialist is unenlightening, and a good non-chemical discussion of the principles of cellular differentiation in relation to carcinogenesis (Rusch). The reason for such homage to non-chemical embryology amidst this pure dedication to biochemistry is difficult to understand.

To turn, however, to the meat of the criticism. What *quality* has this venture as a book? For a book, unless we abandon all cultural regards, is more than a collection of articles bound by a cover. It has a purpose—and indeed, this one claims to—beyond a physical packaging of essays. To what degree then, and with what excellence, has this volume fulfilled its publicized claim of providing for "the orientation of nonspecialists"? To distill the essence of ten years of biochemical research is a formidable and exciting challenge which goes well beyond the job of selecting 27 topics. To be sure, the topics selected are fine, but there is no trick to culling pretty flowers from a well-tended garden. What sort of arrangement do we have in this volume other than mere collection? A careful avoidance of duplication is about the only answer. What efforts were made to have the writers convene and discuss the choice of topics, and how much critical interchange followed the submission of manuscripts? The reviewer is not in a position to know, but if there were any such meetings they must have been hurried and superficial for there is no evidence of effective consultation. Is scientific knowledge so fleeting that time forbade meeting, arguing, designing, and redesigning individual contributions so that a *book* and not another of the scores of bound collections regularly put on the market emerged? Too many "books" are already distinguished from one another by the particular combination of essays they contain. In one case, it is nuclear physiology plus adaptive enzymes; in another, it is adaptive enzymes plus electron transfer. There is something approaching the Hollywoodian in the publishing business: pick a list of big enough stars, and any script will do.

Time and again we are warned of the downgrading of English in scientific writing, and just as often we are reminded that scientific and literary competence are independent and separate gifts. Certainly, it is one thing to demand even of book-writing scientists an elegance of style, but it is another matter to expect scientists to show a respect for it. A book such as this is the product of academic culture, and its composition should at least manifest a regard for basic literary values. Yet, on the fly-leaf we are greeted with the commercial "Not a revised edition of the original . . . but an entirely new book." What thinking person cares whether it's revised or entirely new? Quality, not novelty, is the scholar's search. It is sad that we are expected to respond to this blurb as prospective car buyers are to the ads for the *all new* car. And what passes on the fly-leaf has its match in the interior. There is no use made of a competent literary person who, in consultation with many of the scientists, could give the book some grace. We are forced to swallow, or choke on, phrases like "explosive birth," or "operationally diverse disciplines"; and introductory sentences like "any summary assessment of their role is fated to be overmiserly in description and hazardous in extrapolation," or "the field of enzyme chemistry has evolved from a tiny island in the realm of biochemistry to such vast and heroic proportions that it now begins to overwhelm the field itself by virtue of its almost universal applicability."

Too often in our writing and in our experiments we forget that truth is not so obviously placed nor so unromantically set as to be indifferently chased and caught by the fastest research runner. Much of our biochemistry is research running, and to the reviewer's disappointment, the publication of this book is one more instance of the race. What was a golden opportunity and what could have been a mature reflection by leading biochemists on the direction biochemistry is taking has dwindled to the stature of just another volume of articles, excellent, good, and fair. These, students will seek to acquire by writing for selected reprints and librarians will seek to house by a further squeezing of the books on their already cluttered shelves.—H. STERN, *Chemistry Division, Science Service, Department of Agriculture, Ottawa, Ontario, Canada.*

PLANTS IN ACTION. A Laboratory Manual of Plant Physiology.—L. Machlis & J. G. Torrey. W. H. Freeman & Company, San Francisco. 288 pages. 1956. $3.75.

This new manual presents many interesting features. The inclusion of explanatory text unifies and should make the experiments more meaningful to the student. The organization of the material enables

him to start long-term experiments at the beginning of the semester, leaving the relatively short-term metabolism experiments till later. In this way material that is tangible is covered first and leads into the more abstract. Experiments on plant growth are followed by a group on mineral nutrition. This is followed by water relations. The fourth division of the manual is the most extensive and covers various aspects of cellular metabolism and energy relations.

The experiments themselves are comparatively simple and illustrate different points with little duplication. Many classical experiments, long the mainstay of plant physiology courses, have been put into a modern context.

The manual is amply filled with the now famous Evan Gillespie touch, although one wonders whether some of the illustrations such as the "view of plant physiology laboratory bench" and "corner of greenhouse with bench" are not superfluous.

Among the valuable features of the manual are the appendices including for each experiment a time table and key giving all the materials needed, lists of plant materials, equipment and reagents, and an index. These should make the lot of the laboratory instructor less harried.

About half of the manual consists of data report sheets and questions with spaces for the answers. The format enables rapid grading, especially with large classes. There may be some instructors, however, who will find the prepared questions too limiting for their own group of students and therefore have little use for this section. These preorganized data sheets and questions and the absence of a bibliography of research papers from which some of the experiments have been adapted do not favor the superior and original student.—ALEX SHRIFT, *Botanical Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania.*

PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their services without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. PHYTOPATHOLOGY XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare it. Second Edition. Williams and Wilkins Company, Baltimore, Maryland, 1951.

1. Length. The allowable length of papers is 10 printed pages, with tables, figures, formulae and equations not to exceed 25 % of the total. Authors will be billed at cost for overrun and excess of figures and tables.

2. Format. In the preparation of typescripts, authors are requested to examine a January, 1956, copy of the journal of PLANT PHYSIOLOGY to note particularly the position of headings, general organization, punctuation, abbreviations and the methods of citing literature in order to bring the script into conformity with the general style of the journal. Leave a three inch margin at the top of the first page, liberal side margins and double spacing of lines to permit proofmarking and required editorial changes. Wherever possible metric units and centigrade temperatures should be used. If it is essential to use English units, such as in pounds per acre, include a conversion factor to metric units the first time the English units are used.

3. Headings. Leave all headings unscored and scientific names without underlining, as the editors will proofmark these items in accordance with the practice of the journal. Generic names used alone are usually capitalized but not italicized. In general, do not underscore any lines.

4. Footnotes. Avoid footnotes as far as possible by placing such items under acknowledgments or at end of summary. When footnotes are used, they should be numbered with Arabic numerals.

5. Figures. Copy for figures (photographs and drawings) should consist of 2 sets and should not exceed $8\frac{1}{2} \times 11$ inches. Retain original drawings as a safeguard against loss. Author's name and figure number must be written on the back of each figure. Do *not* put legends into or on the figure, either front or back. Type all legends *double* spaced, properly numbered to correspond to the figures, on a *separate sheet* of your manuscript. Graphs and drawings should be inked with fairly heavy lines and with lettering of approximately uniform size to insure clarity of printed figures after reduction. Photographic prints should be submitted on gloss-finish paper. Limit copy to desired visual details, trim off irrelevant foreground and minimize blank space, as charges for cuts are based on area. The editor will proofmark copy to determine actual size of printed figures.

Authors are asked to group figures whenever possible so that two, three, four or six figures can be reproduced by a single zinc etching. Do not mix half-tone and zinc etchings in the same grouping. Authors of manuscripts can save the journal approximately $1,000 a year by properly grouping their figures.

6. Tables. Place each table on a separate page; do not crowd too much material into a single table. Note the size of the printed page, and that tables should be designed to fit into one column, i.e. 3 inches in width, wherever possible, although full page tables, $6\frac{1}{8}$ inches wide can be used where required. In no case are tables to be printed turned 90° on the page. Use horizontal lines (rules) at top and bottom, but omit or employ such rules sparingly in the interior of the tables. Rules and spaces are usually used in the final printed tables to separate sets of data. For footnotes to tables use the asterisk, double asterisk, dagger, double dagger and section in this sequence as needed. Tabular material should be used sparingly because of its high cost.

7. Acknowledgments. Place acknowledgments at the end of the paper just below summary.

8. Address. The name of your institution and its address should be listed just below the names of the authors.

9. Literature Cited. Use separate pages for the literature cited and type *double spaced*. Give complete citations, author, title, journal name, volume number, inclusive pages and year. (See the literature lists in the January, 1957, issue of the journal for examples.) Punctuate according to the examples, and leave no citations incomplete. Write out names of states in full. Note proper abbreviations of journals. An error frequently noted in manuscripts with long bibliographies is failure to mention in the text all citations listed. Authors are requested to check all citations in the bibliography by careful proofreading of manuscripts prior to submission.

10. Manuscript Review. Manuscripts are sent out for review by two competent reviewers familiar with the field of research covered by the paper. These reviewers may or may not be members of the Editorial Board. In case of marked disagreement between reviewers, the Editor appoints a referee, usually a member of the Board, to make a recommendation for the Board. Usually two months suffices for review and decision of the Editorial Board. Revised manuscripts

should be returned within two months of the authors receiving proposals for revision of their manuscripts, or the revised paper will be published under the date of the receipt of the revised manuscript and not the original date.

11. Galley Proof. Correct all errors clearly and return galley promptly to editor. Authors will be billed at cost for alterations in the galley proofs, other than corrections of printer's errors, involving excessive changes from manuscript copy.

12. Send original copy of manuscript and first carbon to the editor, D. R. Goddard, University of Pennsylvania, Philadelphia 4, Pennsylvania. Retain a carbon copy to insure against loss, and to consult in case of need. Prompt return of revised manuscript copy and galley proof to the editor greatly expedites publication. Write across the top of title page of the manuscript directions for mailing of proof, thus: Mail proof to (author's name and address). If authors wish to have original manuscript or copy for illustration returned subsequent to publication, the editor should be so informed at the time proof is returned.

13. Reprint Order. These should be sent to the editor with corrected galley proof. Late orders for reprints entail extra work and expense by the printer for which charge must be made to the author. Type is destroyed immediately after printing and hence it becomes impossible to supply reprints after publication of papers. Reprint orders should be made out to The Science Press (not to the editor or to the University of Pennsylvania) and sent with *corrected galley* even if formal requisitions or purchase orders for institutional agencies follow later.

14. Abstracts. Authors are requested to submit a typed abstract on the form provided when corrected galley is returned to the editor who will insert proper volume and paging for use by Biological Abstracts.—The Editor.